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Evaluation of Models Used to Assess Effects and Countermeasures Of Microgravity, with Specific Respect to their Utility in Simulating and/or Predicting Space-Related Outcomes

Jacqueline P. Williams
University of Rochester, Rochester, New York

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Jacqueline P. Williams
University of Rochester, Rochester, New York

National Aeronautics and
Space Administration

Langley Research Center
Hampton, Virginia 23681-2199

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EVALUATION OF MODELS USED TO ASSESS EFFECTS AND COUNTERMEASURES OF MICROGRAVITY, WITH SPECIFIC RESPECT TO THEIR UTILITY IN SIMULATING AND/OR PREDICTING SPACE-RELATED OUTCOMES

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Prepared for:

National Aeronautics and Space Administration
Langley Research Center, Virginia

Prepared by:

Jacqueline P. Williams, PhD
University of Rochester
Rochester, New York

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1. Introduction

Within only a few years of Yuri Gagarin reaching low earth orbit (LEO) in 1961, it became apparent that the biological stressors that are imposed on the human body during spaceflight require investigation, with the ultimate goal of preventing, mitigating and/or treating any associated consequences in astronauts, occurring either during or subsequent to spaceflight. These stressors include, but are not limited to, the individual and/or combined effects of extended periods of weightlessness, exposure to space radiation, and acceleration stresses that occur prior to and after a prolonged stay in zero-gravity conditions, amongst others.[1, 2] Decades of international research have been performed since that time; however, as is evident from the conclusions drawn in the most recent decadal survey from the National Academies of Science, Engineering, and Medicine, the majority of the highest priorities required in space research are associated with the effects of reduced gravity.[3]

Due to the looming likelihood of manned missions to the Moon, Mars, *etc*. within the next few decades, it is increasingly incumbent on the National Aeronautics Space Administration (NASA) to gain a deeper understanding of the mechanisms underlying the acute- and long-term biological effects experienced by the human system due to exposure to reduced- and/or micro-gravitational conditions. It is anticipated that, through such an understanding, will come the means of developing countermeasures; however, there are limited opportunities to research such effects under real-time actual exposures. Therefore, there is a significant need to appreciate the best means by which we can decipher the various physiologic consequences of working and/or living in low gravity and, thereby, facilitate future space exploration. This report, funded by the NASA, was undertaken to provide an independent assessment of the major models used in this field to date, as well as their potential effectiveness in closing the gaps in our biological knowledge and enabling countermeasure development against the effects of microgravity.

1.1. Models of Microgravity

The utilization of models, whether to predict risks, identify biological mechanisms underlying effects, or assist in countermeasure development, has long been seen as a critical component in space research.[4, 5] The models considered in this report fall under two overarching categories: human versus animal. These categories are further divided into space versus ground-based studies.

General descriptions of each of the considered model systems (see **Figure 1**), their history and implementation are provided in the next section.

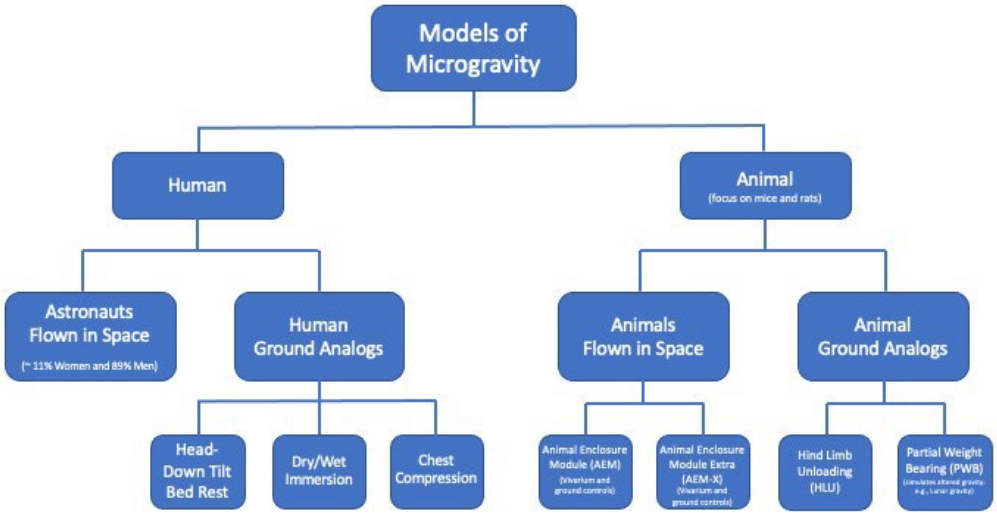

Figure 1. Overview of models of microgravity.

1.1.1. *Astronaut Studies*

Adaptive physiological responses occur during flight and have been observed across the astronaut cohort in all biological systems,[6-10] with the level and extent of adaptation frequently appearing to be dependent on mission length. Subsequent to return to full gravity, a period of "disability" and/or impairment may be seen during the recovery or readaptation period, with the observed symptoms including muscular atrophy,[11, 12] bone demineralization,[11, 12] orthostatic intolerance,[13] and neuro-vestibular changes,[14] as well as a risk of acute and/or delayed musculoskeletal and other issues.[15] Indeed, a critical question in terms of risk estimation is distinguishing between those space-induced changes that are fully recoverable versus those that may persist, leading to long-term risk. Interestingly, as described in a review by Demontis *et al.*,[16] observation of such symptoms led to the development of the field of 'space physiology,' a scientific discipline dedicated to understanding alterations in, and restoration of, organismal functioning under space conditions. Therefore, making a connection between biomarkers and/or intermediate risk factors to the probability of developing chronic diseases has become an important component of the space physiology field, so that direct examination of the changes seen in the astronaut cohort might reasonably be considered the gold standard for use in risk estimation.

1.1.2. *Ground-Based Human Studies*

Although the potential effects of zero gravity on the human body had been the subject of discussion and research for decades,[17] the recognition of rapid adaptive responses seen at the beginning of human spaceflight exposed the need for models of microgravity. In the early 1960s, the buoyancy induced by water immersion appeared to be a logical approach to mimicking weightlessness;[17, 18] however, evidence of rapid onset subacute dermatitis and a negative pressure breathing effect limited its use to short term (6—12 hour) studies only.[19-21] A modification of this model to dry (versus wet) immersion, using a highly elastic, water impermeable material, has been adopted by Soviet and Scandinavian researchers[21, 22] although this technique has been used less frequently elsewhere.

A more widely used ground-based human model is bed rest, which has been utilized in medicine for centuries; the history of bed rest as a tool, together with a description of its associated physiological changes, has been covered in numerous review articles.[23, 24] Indeed, the characterization of bed rest as a model of musculoskeletal disuse predated space travel; beginning in the late 1940s, Deitrick *et al*. examined the response of immobilization in four healthy subjects[25, 26] with the goal of differentiating the detrimental response of bed rest alone from that of disease. The group demonstrated increased nitrogen and calcium excretion, loss of muscle volume and strength, orthostatic intolerance and reduced blood pressure during immobilization, with persistence of some of these symptoms following reambulation; such observations may be key when identifying long-term risk(s). Over time, cosmonaut complaints of sleep issues led the Soviets to further investigate the effect of head-down tilt (HDT) versus horizontal bed rest as a means of simulating the fluid redistributions experienced in space.[27] The testing of various angles of tilt culminated in an almost universal adoption of a -6º head-down tilt, which produces approximately -0.1 Gz whilst still allowing for relative comfort on the part of the participant.[27]

1.1.3. *Animal Studies in Space*

Between the late 1950s and 1970, animals preceded humans into space as part of the effort to develop safe vessels for human spaceflight, with American and Soviet scientists making use of monkeys, chimpanzees and dogs.[28, 29] After 1970, the purpose of animal spaceflight appeared to change, becoming more broadly focused on determining the physiologic risks and effects associated with space travel;[30] these later studies made use of a range of different species, including

rodents, turtles, insects, fish, jellyfish, amoebae and algae.[28] Unfortunately, an amalgamation of circumstances, such as the 1998 closing of Spacelab, the ending of the shuttle program in 2010, and limitations in research capabilities on the International Space Station (ISS) due to budget shortfalls, reduced more recent United States (U.S.) involvement in space animal experiments.[29]

With the growing interest in performing experiments in space came the need to house animals during spaceflight. There has been an emphasis on developing housing for rodents;[31-33] in general, rodents, and mice in particular, are the favored mammalian model in biological and medical research due to their small size, ability to breed easily and mature quickly, and the availability of a variety of strains with different genetic backgrounds.[34-36] However, in contrast to the relative standardization of ground-based laboratory conditions, the constraints of spaceflight in terms of limited payload and housing with automated life support and maintenance systems has led to the need for non-standard rodent housing facilities in space.[37] In the late 1990s and early 2000s, through a collaboration between the NASA Ames Research Center and the General Dynamics Company for the Student Shuttle Flight Program, the Animal Enclosure Module (AEM) and Animal Enclosure Module Extra (AEM-X) were developed.[38] The AEMs offered small rodent housing that supported 5—8 rats (or 6—10 mice) for up to 30 days on shuttle flights;[39] this platform then was used as the basis for the NASA's Rodent Research Hardware system (RRHS), an ISS research facility.[40] The RRHS has three major components: the Transporter, which offers housing for rodents during transport to and from the ISS; the Rodent Habitat, which provides in-orbit housing; and the Animal Access Unit (AAU), which is the interface between the Habitat and Transporter for the transfer of the animals between the units and for access to the animals for science operations.[40, 41] In parallel with the creation of the AEM system came the development of the Mouse Drawer System (MDS), a facility funded by the Italian Space Agency to support microgravity research into bone changes and osteoporosis on the ISS.[42, 43] The MDS can hold six mice in individual cages, allowing for olfactory, but no physical, contact among the animals. However, despite these developments, there remains a limited availability of animal habitats on the ISS.[29]

1.1.4. *Ground-Based Animal Studies*

Animal analogs have long been utilized in the investigation of pathological and physiological changes seen in humans, particularly in response to conditions that are difficult or unethical to

simulate in humans.[44, 45] With respect to space, such models have helped us to gain insight into the potential mission-critical and post-mission effects of spaceflight in tissues, organs, biological systems, and functional performances of astronauts.[46, 47] Currently, the gold standard for ground-based microgravity research is considered to be the rodent hind limb unloading (HLU) model, originally created to investigate the relationship between microgravity and musculoskeletal outcomes,[48, 49] but now being used to assess a much broader range of physiological responses.[46] As described by Morey-Holton and Globus,[49] during the development of the HLU model of simulated weightlessness, a number of physiological responses (differential muscle atrophy;[50] cephalad fluid shift[51]) and experimental conditions (freedom to eat, move and groom; unloading of the hindlimbs without paralysis, enabling recovery; normal weight gain in growing animals) needed to be invoked.[49] It is worth noting that alternative models of muscular disuse, such as hind limb immobilization[52-54] and neurectomy/nerve crushing,[55] do not result in the cephalic fluid shifts and/or differential muscle atrophy, respectively, seen with spaceflight.

The mechanics of the HLU model involves lifting the hind limbs of an animal, usually a rodent, off the cage floor, typically holding the torso at a 30^{o} angle; importantly, the hind limbs must be kept from touching the floor whilst allowing normal mechanical loading of the fore limbs. The first documented use of HLU[48] involved suspending rats via an orthopedic harness that was bonded to the back of the rodent. The apparatus consisted of a freely rotating fish line swivel on an overarching aluminum beam that allowed the rats to both turn in a 360^{o} arc and navigate across the cage to reach food and water.[56] Evolution of this technique by Morey-Holton and Globus[49] involved wrapping the base of rodent tails with orthopedic traction tape or foam, followed by connection to a clip attached to a pulley system, enabling the hind limbs to be lifted and adjusted over time. Multiple further modifications to this protocol have occurred over the years:[57-61] some groups, such as Wilkerson *et al*.,[62] opted for a plastic tail cast, with hooks on either end of the cast, connected by a chain to a swivel apparatus; Woodman *et al*. used a triangular-shaped wire, sandwiched between two layers of vinyl cloth, glued to the proximal two-thirds of the tail to lift the hind limbs;[63] Zhang *et al*. taped a plastic bar laterally to the proximal portion of the tail, which was secured by more tape twined around the tail and wire netting.[64] In a move away from these relatively non-invasive methods, Ferreira *et al*. used a surgically implanted steel wire ring that passed through the 5th, 6th or 7th intervertebral disc space.[65] However, despite these modifications, the protocol described by Morey-Holton and Globus[66] remains the most frequently used.

In addition to the weightlessness experienced in space *per se*, planetary exploration will likely lead to exposure to weaker gravitational loads relative to Earth; for example, the Moon has 16% of the gravity of the Earth, whereas Mars has 38%.[67, 68] To investigate outcomes from reduced versus zero gravity, a model of partial weight bearing (PWB) or suspension (PWS) has been developed using a two-point body harness, which allows for adjustable quadrupedal unloading[69] without the cephalad fluid shifts; this harness has been adapted for use in both mice[70] and rats.[68, 71] The apparatus differs from that of Morey-Holton and Globus[66] since it not only involves a tail wrap, but also a jacket that supports the front limbs. The jacket and the tail wrap are connected by an adjustable bead chain and spaced by a hollow metal rod to distribute load, with the cage lid preventing rodents from climbing and a wheel with linear freedom across a rail allowing for cage exploration.[69] Promisingly, studies to date have demonstrated a strong correlation between musculoskeletal outcomes and the degree of partial weight bearing.[72, 73] Correlations with other endpoints of interest are included in this report.

2. Effects of Microgravity – Methodology

2.1. Literature Scoring System

As our evaluation of the various models began with respect to their contribution to current understanding of the various effects of microgravity, it quickly became apparent that a large literature database is available across the various endpoints being considered. However, a systematic and cohesive approach to cross-evaluation of the various models was found to be problematic: multiple methodologies were utilized within each model system; a broad choice of endpoints was evaluated within each disease category; and a variable spectrum of experimental conditions were used within each ground-based study to simulate the space environment. This made direct cross-comparisons among models difficult.

It was determined, therefore, that as a first step, in order to place a priority level on the relevance of each publication, a quality rating was developed. The methodology adopted was a modified scoring system, based on that employed by the Electric Power Research Institute[74] and the National Council for Radiation Protection and Measurement[75] in recent reviews of the literature with respect to the effects of radiation on the eye. The system assigned scores according to the inclusion or absence from each study of the following criteria:

Experimental Space Environment Conditions (Physical/Dietary/Psychosocial):

- Acceleration stress from leaving/returning to Earth: present = +1; absent = 0
- Microgravity simulation: head-down model = +2; prone/PWB = +1; limb immobilization = 0
- Radiation: galactic cosmic ray (GCR)-simulation/mixed field = +2; single ion/low dose rate gamma = +1; low dose gamma = 0; >1 Gy/high dose rate radiation = -1
- Raised CO_2 levels: present = +1; absent = 0
- Social isolation = +2; small group housing = +1; large group housing (>5)/no details = 0
- Limited/controlled diet = +1; no details = 0

Cohort Characteristics:

- Sex: comparison of gender effects = +2; single gender/both grouped = +1; no details = 0
- Fitness assessment: performed = +1; not assessed/described = 0

- Age relevance to astronaut cohort: relevant (~45 years) = +2; out-of-range (<30 yrs, young adult) = +1; no details = 0; juvenile = -1

Data/Statistical Quality:

- Group size: ≥10 = +1; ≤10 = 0
- Use of control groups: space habitat + microgravity vs. space habitat – microgravity (vs. vivarium) = +3; age-matched +/- microgravity +/- additional space stressor = +2; age-matched +/- microgravity = +1; internal control only = 0
- Time points: baseline = +1; flight equivalent: single = +1; multiple = +2; recovery acute R0—R10): single = +1; multiple = +2; recovery mid-term (R10—R30): single = +1; multiple = +2; recovery long-term (>R30): single = +1; multiple = +2

Subsequent to the development of the scoring system, a literature search was performed using relevant keywords for each model/category. As a result of the search, over 400 publications were assessed directly using the system: 95 publications were scored under the astronaut category; 105 in the ground-based human studies category; 71 publications covered studies of animals flown in space; and 130 publications addressed ground-based animal studies. Following scoring, publications were sorted by subject matter/endpoint; the top scoring papers are listed in tables in each relevant section. Of note, where a publication described multiple studies that were deemed sufficiently independent, the sub-studies were scored individually. Furthermore, given multiple indications of dependence on mission length, the publications were subdivided with respect to duration. For astronaut (and animals in space) studies, the threshold for short-duration was seen as ≤30 days flight, with long duration flights considered as ~4—6 months. In contrast, ground-based studies, both human and animal, were divided into short-term (≤10 days), medium-term (10—30 days), and long-term (≥50 days). The contrast in the temporal threshold used for "long-term" in the space- versus-ground-based studies has been based on limited musculoskeletal findings that showed, for some parameters (*e.g.* trabecular bone loss and recovery), 60—90 days of bed rest[76] appears to recapitulate the long-term deficit dynamics seen after 6-months of spaceflight.[77]

We recognize that the methodology used is not comprehensive and that many publications were not included in the scoring process due to time constraints, accidental omission, or limitations on public access. However, it is important to note that, although use of this system allowed us to

focus on a slightly narrower database of publications, all accessed publications were considered during the writing of this report and their data included, as and where appropriate.

3. Musculoskeletal Outcomes

3.1. Publications Overview

3.1.1. *Astronaut Category* (**Table 1**)

- Of the 29 papers directly assessed in the astronaut-musculoskeletal category, only one considered sex as a variable in its initial findings,[78] even though females made up 16% of the participants across all of the studies; of note, this participation rate is roughly equivalent to the gender makeup of the astronaut workforce. Interestingly, in general, few research studies have addressed sex as a factor in the musculoskeletal area,[79, 80] despite a well-accepted sex differential seen in various aspects of the musculoskeletal system.[80-82]
- Not surprisingly, given the numbers of astronauts that fly per mission, it was rare for the statistical group size (*n*) to reach the imposed statistical threshold limit of ten, and this was true for all of the astronaut categories considered in this report.
- Although roughly half of the studies considered late time points tracking musculoskeletal changes over the first year of recovery, only four performed inflight analyses.[83-86]

3.1.2. *Ground-Based Human Category* (**Table 2**)

- The overall scores for the 31 human ground-based immobilization publications were relatively lower than those for the astronaut studies, despite the potential for larger group sizes, *etc*. These scores were driven chiefly by a failure to recapitulate the majority of stress conditions found in the space environment, with controlled diet being one of the few additional parameters imposed with any frequency.
- Surprisingly, the majority of studies surveyed in this category used males alone as participants (20/31). One HDT study[87] considered sex as a variable in its analysis, although two limb immobilization studies performed a head-to-head comparison looking at gender effects on muscle atrophy.[88, 89] Furthermore, given the inherent physical training requirements embedded in selection of the astronaut cohort,[90, 91] only 13 of the studies provided details of the physical fitness of participants.
- Although all but a few of the scored publications performed interim analyses during immobilization, only six of the studies looked at musculoskeletal changes beyond the first few days/weeks of reambulation.[92-97]

3.1.3. *Animals in Space Category* (**Table 3**)

- All 23 studies considered in this category used rodent models, with a ratio of 7:16 rat to mouse subjects; all but one of the murine studies used a strain on a C57BL/6 (inbred) background.
- With respect to simulating astronaut characteristics (and ignoring arguments that could be, and have been, made with respect to the relevance of the mouse to the human for musculo-skeletal biological/mechanistic studies[98, 99]), the majority of the animal space studies made use of juvenile mice, with only 3/23[100-102] using appropriately-aged six-month old animals, and an additional 2/23 using young adults.[103, 104] Indeed, Ghosh *et al.*[101] noted that their observation of differential results between studies performed on different shuttle missions may have reflected the (lack of) skeletal maturity in mice less than 5—6 months of age.[105] All studies used single gender groups (13:10; female:male) and none considered sex as a factor.
- Off-setting the limited simulation of astronaut characteristics, many of the studies were performed using multiple control groups, including both age-matched cohorts maintained under replica housing conditions to those used in space (*e.g.* AEM), as well as a matched vivarium cohort, potentially enabling broader and more direct comparisons in the context of diverse environmental parameters.
- The logistics of performing prolonged animal studies in space resulted in only a single scored study[106] including a flight time beyond 30 days; unfortunately, inflight mortality in this specific study also led to group sizes ($n = 2$) that did not support statistical evaluation. Finally, despite many of the outcomes of interest being long-term in nature, only two studies performed postflight analyses beyond the first week of recovery.[107, 108]

3.1.4. *Ground-Based Animal Category* (**Table 4**)

- The majority of the 64 studies considered in this category used the hind limb unloading (HLU) model, with 12 using partial weight bearing (PWB)[68-73, 109-114] and two using immobilization.[53, 54] All of the considered studies made use of rodent models, with a ratio of 31:33 rat to mouse subjects; all but one of the HLU studies used the C57BL/6J strain, although four of the PWB investigations used Balb/c.[70, 111, 113, 114]
- Despite the relative experimental freedom available to ground-based versus spaceflight animal researchers, overall, there has been limited effort to recapitulate the broader space environment as part of microgravity studies. Of the publications considered in this category,

17 administered radiation as an additional stressor, but none included hypercapnia. In roughly half of the studies, subjects were socially isolated, although this likely was a result of the technical aspects of the HLU/PWB apparatus rather than a deliberately imposed condition; of note, actual isolation rates may have been higher since the social housing conditions were not always included in the experimental details.

- As noted in **section 3.1.1.**, there was little interrogation of an effect due to sex, with 51/64 of the scored publications using male subjects alone; only two studies specifically compared the responses of males to females.[115, 116] 8/64 of the studies used age-appropriate (~six months old) animals, with 24 using juveniles.
- 12 of the HLU studies performed interim analyses during unloading[58, 115-125] (along with 5/12 PWB studies)[67, 68, 71, 72, 110] and, despite the long-term persistence of musculoskeletal changes seen in astronauts, only six of the scored studies looked at time points beyond the first few weeks of recovery.

Table 1: Highest scoring publications in the astronaut musculoskeletal category.

ASTRONAUT	Environment parameters	Astronaut characteristics	Science / time points	Score	Main Scientific Findings
Short flight (≤30 days)#					
Pool-Goudzwaard AL *et al*. 2015[83]	9	3	7	19	12—15 days flight compared to bed-rest: Prevalence in astronauts with history of lower back pain. Pain was self-limiting (≥9 days inflight)
Tesch PA *et al*. 2005[126]	9	3	5	17	16 days flight compared to matched ground: Significant decrease in maximal voluntary isometric, concentric and eccentric knee extensor force at recovery day 1. Normalized by recovery day 16
Long flight (≥4 months)					
English KL *et al*. 2015[127]	9	4	5	18	~163 days flight: Mean isokinetic strength declined postflight, with persistence
English KL *et al*. 2020[128]	9	4	5	18	~165 days flight: Femoral neck BMD†, knee extensor peak torque, cone agility test time and VO_{2peak}†† decreased inflight. High intensity/low volume exercise attenuated/ stopped loss of some parameters
Sibonga J *et al*. 2019[85]	9	2	7	18	154—173 days flight: Comparison of ARED* ± bis-phosphonate; additive effect on attenuation
Burkhart K *et al*. 2019[129]	9	2	6	17	4—7 months flight: Paraspinal cross-sectional area and attenuation decline/persist after long-duration spaceflight
Vico L *et al*. 2000[130]	9	3	5	17	6 months flight: Cancellous and cortical bone loss occurs in weight-bearing bones (tibia) within 1—2 months flights. Progress with mission length; persists postflight
Sibonga JD *et al*. 2007[131]	9	3	5	17	~173 days flight: Average bone loss across all sites 2—9%; modeled recovery of 50% within 9 months
Vico L *et al*. 2017[132]	9	3	5	17	4—6 months flight: Tibial cortical porosity and trabecular bone fail to recover within 1-year postflight. Decline of remodeling markers after 6-months recovery

Short flights have been defined as 30 days or less[9]

† BMD: bone mineral density

†† VO_{2peak} is the peak rate of oxygen consumed during exercise and looks at both cardiovascular and skeletal muscle oxygen function

* ARED: Areal resistive exercise device

Table 2: Highest scoring publications in the ground-based human musculoskeletal category.

HUMAN HDT STUDIES	Environment parameters	Astronaut characteristics	Science / time points	Score	Main Scientific Findings
Short-term (≤10 days HDT)					
Mulder E *et al*. 2015[133]	3	2	7	12	5 days HDT: 2—3% loss of knee plantar and extensor CSA†. 5 days deemed insufficient for assessment
Baecker N *et al*. 2003[134]	3	2	5	10	6 days HDT: Metabolic study with controlled diet, showed rapid rise in osteoclast activity
Medium-term (10—30 days HDT)					
Belavý DL *et al*. 2011[92]	2	3	7	12	21 days HDT: Cortical area and thickness decreased in tibia (vs. radius) over 30 days; progressed over HDT 60. Recovery began days 3—15; complete by R90
Morgan JL *et al*. 2012[135]	3	2	7	12	30 days HDT: Assessment of urinary markers of bone metabolism demonstrated short-term changes
Zwart SR *et al*. 2007[136]	3	2	5	10	30 days HDT: Femoral shaft and hip BMD decreased with bed rest; mitigated with exercise
Liphardt AM *et al*. 2018[137]	3	3	4	10	21 days HDT: Changes (decreases) in cartilage biomarkers during bed rest: COMP, MMP-3 and MMP-9††
Smith SM *et al*. 2009[138]	3	2	5	10	21 days HDT: Time-related decrease in hip and trochanter BMD and total body BMC†††
Long-term (≥50 days HDT)					
Rittweger J *et al*. 2010[93]	2	2	8	12	56 days HDT: Greatest bone loss in tibial distal epiphysis (2% on bed rest day 55). Persistent to R1 year. Abrogated with exercise
Belavý DL *et al*. 2011[92]	2	2	8	12	60 days HDT: Cortical area and thickness decreased in tibia (vs. radius) over 30 days; progressed over 60 dys. Recovery began days 3—15; complete by day 90
Beller G *et al*. 2011[95]	2	3	6	12	60 days HDT: BMD measured up to 1 year post-HDT. Greatest loss in distal tibia and proximal femur. Loss remained at 1 year – no mitigation by countermeasures
Austermann K *et al*. 2021[139]	3	2	7	12	60 days HDT: Bone turnover markers showed increase in bone resorption, no change in formation. No effect from countermeasure

Shackelford LC 2004[87]	2	4	5	11	17 wks HDT: Loss (1—9%) of lumbar spine, hip, pelvis, total body BMD. Mitigated with resistive exercise
Miokovic T *et al*. 2012[96]	2	2	7	11	60 days HDT: Long recovery assessment of muscle atrophy showing differential atrophy dependent on muscle
Armbrecht G *et al*. 2010[94]	1	2	7	10	56 days HDT: Markers of bone resorption/formation increased or decreased respectively.
Rittweger J *et al*. 2005[140]	3	3	4	10	90 days HDT: Decrease in calf muscle CSA and tibial BMC. Effects differentially mitigated by exercise
Zerwekh JE *et al*. 1998[141]	3	2	5	10	12 weeks HDT: Using multiple parameters, demonstrated rapid and sustained increase in bone resorption; more subtle effects (decline) on bone formation
Rittweger J *et al*. 2009[97]	3	2	5	10	90 days HDT: Focus on recovery: bone and muscle recovery over 100—150 days, followed by overshoot

† CSA: cross-sectional area

†† COMP: cartilage oligomeric matrix protein; MMP: matrix metalloproteinase

††† BMC: bone mineral content

Table 3: Highest scoring publications in the space animal musculoskeletal category.

ANIMAL SPACE STUDIES	Environment parameters	Astronaut characteristics	Science / time points	Score	Main Scientific Findings
Short flight (~30 days)					
Jee WS *et al*. 1983[107]	9	0	6	15	19 days spaceflight decreased mass of mineralized tissue and increased fat content of bone marrow in proximal tibial and humeral metaphyses
Wronski TJ *et al*. 1983[108]	9	0	6	15	19 days spaceflight decreased periosteal bone formation in tibia, humerus diaphysis and rib
Coulombe JC *et al*. 2021[102]	7	3	5	15	2—3 weeks spaceflight: Compared 9-wk to 32-wk old mice. Increased age-associated effects: trabecular vs. cortical bone loss; overall skeletal bone loss
Fitzgerald J *et al*. 2019[103]	8	2	4	14	30 days spaceflight induced differential effects in articular vs. sternal cartilage, supporting role of microgravity on changes in weight-bearing tissues
Cavolina JM *et al*. 1997[142]	7	0	6	13	Hormone loss (ovariectomy) exacerbated periosteal bone formation after 14 days spaceflight
Zhang B *et al*. 2013[100]	7	3	3	13	15 days spaceflight induced bone loss in non-weight-bearing bones (calvaria)
Ghosh P *et al*. 2016[101]	7	3	3	13	Age-related (23 vs. 9 weeks old) mandibular bone loss following 15 days spaceflight; noted role of alternative environmental factors
Long flight (~ 3 months)					
Tavella S *et al*. 2012[106]	9	0	4	13	3 months: Enhanced bone resorption in both wild-type and PTN† transgenic mice over 90 days spaceflight

† PTN: pleiotrophin

Table 4: Highest scoring publications in the ground-based animal musculoskeletal category.

GROUND-BASED ANIMAL STUDIES – HLU	Environment parameters	Astronaut characteristics	Science / time points	Score	Main Scientific Findings
Short-term (<10 days)					
Morris CA *et al*. 2005[143]	5	3	2	10	3, 7 days HLU: Use of Bowman-Burk inhibitor to reduce protein degradation attenuated muscle loss
Yumoto K *et al*. 2010[144]	5	2	3	10	7 days HLU: Suppression of osteoblastogenesis and increase in osteoclast numbers were accelerated by exposure to low dose heavy ions
Medium-term (10—30 days)					
Shirazi-Fard Y *et al*. 2014[145]	5	3	9	17	28 days HLU induced loss of trabecular BMC and vBMD†. Exercise enhanced recovery. Pre-loading exercise did not affect changes due to 2º HLU
Shirazi-Fard Y *et al*. 2013[146]	5	3	9	17	Two 28 days HLU with 56-day interim recovery did not show exacerbated negative effects or impede recovery
Shirazi-Fard Y et al. 2013b[147]	5	3	7	15	Following 28 days HLU, recovery in rat PTM†† more closely resembled discordant dynamics seen in astronaut proximal femur (versus rat femur)
Tou JC *et al*. 2005[123]	5	2	6	13	38 days HLU: Comparing purified vs. unpurified diets showed differences in urinary markers (corticosterone, calcium), bone lengths, etc.
Cunningham HC *et al*. 2018[148]	4	3	6	13	14 days HLU: Bone loss in adult (9 months) versus aged (28 months) rats. Aged rats showed delay in adaptive response, with slower recovery.
Mortreux M *et al*. 2021[116]	4	3	5	12	14 days HLU: Detailed assessment of muscle loss comparing male to female response. Conclusion: females sustain less muscle deconditioning
Alwood JS *et al*. 2010[149]	5	2	4	11	Acute 0.5 Gy dose ^{56}Fe ions (day 11) did not alter effects of 14 days HLU (reduced cancellous bone fraction [-14%]; thinned trabeculae [-9%]; increased SMI††† [+129%]); possible persistent SMI changes
Ghosh P *et al*. 2016[150]	5	2	4	11	Acute 1 Gy dose ^{56}Fe ions (day 3) exacerbated loss of gastrocnemius muscle mass induced by 13—16 days HLU. HLU reduced trabecular thickness
Ferreira JA *et al*. 2011[65]	2	3	6	11	14/28 days HLU: Alternate HLU model. Significant and progressive soleus atrophy following tail-ring HLU

Childress P *et al*. 2018[118]	5	0	5	10	28 days HLU including launch simulation: Repair of induced fracture prior to HLU was impaired (52% reduction in callus volume) versus controls
Delong A *et al*. 2020[119]	3	2	5	10	3 weeks HLU: Tibial compression, applied 4 times per week, mitigated loss of cortical and trabecular bone
Krause AR *et al*. 2020[151]	5	2	3	10	2 weeks HLU: Tibial compression applied post-suspension improved bone, but not muscle, recovery
Krause AR *et al*. 2017[152]	5	2	3	10	14 days HLU: Changes in BV/TV*, trabecular number and mineral density exacerbated by proton/O_2 irradiation; no effects on changes in muscle mass
Lloyd SA *et al*. 2012[153]	3	2	5	10	4 weeks HLU: Combined HLU and 1 Gy protons: additive response in trabecular and cortical bone loss
Thomason DB *et al*. 1987[125]	4	1	5	10	28 days HLU: Time course of recovery of soleus; differential recovery of proteins
PARTIAL WEIGHT-BEARING (PWB)					
Medium-term (10—30 days)					
Mortreux M *et al*. 2019[73]	4	2	5	11	30 days PWB with variable unloading (70%, 40%, 20%) demonstrated load- and time-related deficits in multiple parameters, including muscle CSA
Mortreux M *et al*. 2020[109]	4	2	4	10	28 days PWB with variable unloading (100%, 70%, 40%, 20%): No significant modifications in blood pressure, stress markers; dose-related increase in plasma corticosterone
Mortreux M *et al*. 2019[110]	4	2	4	10	14 days PWB: Resveratrol mitigated muscle deconditioning and atrophy
Macias BR *et al*. 2016[111]	5	1	4	10	21 days PWB: Combined PWB and low dose heavy ions reduced bone formation and increased bone resorption

† vBMD: volumetric bone mineral density
†† PTM: proximal tibia metaphysis
††† SMI: structural model index
* BV/TV: absolute trabecular and cortical bone volume fraction

3.2. Musculoskeletal Microgravity Studies: Data Comparisons Across Models

As noted in the **Introduction**, some of the first impairments that were observed in astronauts were recognized in the musculoskeletal system. Therefore, not surprisingly, musculoskeletal change is one of the most widely studied physiological areas in the space research population, with the main foci of interest being bone loss or demineralization[10, 12, 14, 154] and muscle loss/atrophy in terms of both volume and strength;[12, 155] both have been associated with changes in metabolic and microenvironmental homeostasis.[156, 157]

3.2.1. *Bone Effects*

3.2.1.1. *Bone-Associated Outcomes*: Multiple studies across astronaut mission programs have consistently indicated that long-term spaceflight is associated with a reduction in bone mineral density (BMD).[86, 130, 131, 158] This loss has appeared greatest in the bones that are predominantly involved in supporting the body's weight against gravity (*e.g.*, lumbar spine, femoral neck, pelvis, *etc.*),[16] preferentially affecting trabecular versus cortical bone,[130, 132, 159, 160] with an associated increase in the potential risks of cervical and lumbar intervertebral disk herniations,[15, 161] fracture,[159, 162] and aggravated lower back pain.[83, 161]

Assessing bed rest as an analog for spaceflight, and focusing chiefly on those studies using HDT, multiple studies and reviews have described outcomes in HDT study participants related to bone loss that overlap with those seen in astronauts.[24, 76, 163, 164] These include a decline in areal BMD (aBMD) in the lower skeleton,[87, 92, 93, 95] changes in calcium metabolism,[87, 136, 141, 165] and a shift in osteoblast-osteoclast kinetics.[94, 141, 166] However, although bed rest studies have generally reported a differential (greater) loss from trabecular versus cortical bone,[92, 167] Cervinka *et al.* pooled data from several bed rest studies of varying lengths (24—90 days) and, contrary to the astronaut data, suggested that there was initial preferential bone loss from the cortical compartment, with accelerated trabecular loss seen only after 60 days of disuse.[168]

Despite the presumption of a differential response to gravity due to a quadrupedal conformation, the majority of the scored rodent space studies also have demonstrated changes with respect to bone loss in the hind weight-bearing limbs, with a reduction in bone density in the lumbar vertebrae,[169] pelvis,[104] and dorsal long bones,[170, 171] differential (greater) trabecular versus cortical depletion,[102, 170, 171] and increased osteoclast-mediated bone resorption.[104, 106, 169, 170] However, limitations on flight times and some experimental (principally hormonal) manipulations

have resulted in heterogeneous results.[142] The scored ground-based HLU rodent studies also demonstrated bone loss from weight-bearing long bones,[69, 172-175] differential trabecular and cortical effects,[72, 172] and increased osteoclast-mediated bone resorption;[120, 173, 176] significantly, these outcomes are consistently exacerbated when HLU is combined with radiation,[149, 173, 176, 177] including the use of space-relevant low doses.[152] Also worth noting is that several PWB studies have indicated that trabecular bone deterioration was singularly affected by whole body suspension, with effects proportional to the degree of PWB.[68, 69, 72] Importantly, Macias *et al*., using combined studies of PWB and irradiation, demonstrated differential effects on outcomes when using fractionated sparsely ionizing radiation versus fractionated heavy ions; for example, fractionated X-irradiation was seen to protect against radiation-induced bone loss whereas fractionated heavy ion (^{28}Si) irradiation exacerbated the effect seen following an acute exposure.[111] Such observations emphasize the need to accurately simulate space radiation in terms of both dose and dose rate.

3.2.1.2. *Time Line of Progression*: A 2017 NASA Evidence Report indicated that the average rate of aBMD loss in astronauts is 1—1.5% per month in space,[178] although the authors noted a high degree of variability, both among skeletal bone sites and between individuals. Indeed, analysis of combined data from both American and Soviet/Russian programs by LeBlanc *et al*. showed significant variations in bone loss dependent on the analytic technique used (*e.g*. dual-energy X-ray absorptiometry [DXA] vs. quantitative computed tomography [QCT]) and the skeletal area measured.[179] Nonetheless, a recent meta-analysis of data from 25 publications, covering ~190 astronauts across several space programs, supported a bone loss rate of 0.8% per month.[180] Interestingly, supporting the role played by analytic tool choice, work from Lang *et al*., looking at data from 14 ISS members, indicated a similar rate of aBMD loss at 0.9%/month from the spine versus 1.4—1.5%/month from the hip, although the alternative use of volumetric BMD (vBMD) suggested a 1.2—1.5%/month loss from spine versus 2.2—2.7%/month from the hip.[158]

In addition to uncertainty regarding the incidence rate, the time line for the progression of the bone loss changes is unclear.[178] Few astronaut studies have performed inflight analyses, with most restricted to analysis of bone turnover markers found in urine and sampled at later time points.[84-86] One inflight *in vitro* study suggested that changes may begin as early as day 17,[181] whereas the above-mentioned meta-analysis suggested that bone resorptive markers increased

hyperbolically, with a time to half-max of 11 days and a plateau at 113%.[180] In contrast, one study showed no change in urinary bone formation markers during the first 30 days of flight, but subsequently increasing at a rate of 7% per month.[180] Overall, despite a lack of absolute confirmative data, as noted in a recent review,[182] it appears reasonable, for risk estimation purposes, to assume that bone loss begins immediately upon entry into weightlessness.

Human ground-based studies have been plagued by the same experimental uncertainties seen in astronauts regarding individual and technological variability.[164, 183] Nonetheless, HDT bed rest studies have consistently indicated a slower rate of bone loss compared to astronauts, with estimates varying from 0.3% to 1% per month.[24, 76] In parallel, investigators have seen a slightly slower rise in bone resorption markers within the first 1—2 weeks of starting HDT,[138, 139, 184] although at least one study indicated an increase in resorption markers as early as day four.[134] In general, bone resorption appears to increase only by 50—75% during bed rest versus the 100—150% change seen in astronauts during flight.[135] In contrast, bone formation markers have remained relatively unchanged across a number of the ground-based study periods.[135, 138, 139, 184] As an aside, one study using horizontal bed rest and comparing older (~60 yrs) versus younger (~23 yrs) males indicated that, although baseline bone metabolic markers were lower in the older cohort, the relative rise in resorption was less and there was no effect on bone formation, leading the authors to suggest that older individuals may be at reduced relative risk of bone loss following disuse.[185]

Turning to the rodent models, we were unable to identify spaceflight studies that performed inflight analyses of musculoskeletal changes, so that rate of bone loss and the time line of changes in the space rodent model appears undetermined at the present time. Worth noting is that two early rat studies showed that, at the first recovery time point (recovery day zero), both spaceflight and the corresponding ground-based synchronous controls demonstrated greater bone loss relative to the vivarium cohort, suggesting that at least some of the observed effects may have been stress- versus microgravity-induced.[107, 108] Of the few scored ground-based HLU studies that undertook interim (mid-HLU) analyses, Delong *et al*. performed serial scanning in mice using micro-computed tomography (μCT) and demonstrated a mean -30% change in trabecular bone microstructure by day 11 of HLU, with further progression to a -51% change by day 21.[119] In alignment with astronaut studies, cortical bone loss was lower, calculated at -1% and -6% on days 11 and 21, respectively. These data supported earlier findings from the same group, which had

indicated changes in trabecular bone by day seven of HLU, whilst loss in cortical bone was not seen until day 21.[58] However, as noted in a recent review, the magnitude of bone loss varies significantly across studies,[46] a potential consequence of variations in methodologies, endpoints, study duration, subject age (skeletal maturity), sex and strain. For example, Cabahug-Zuckerman *et al*. failed to show any changes in either trabecular or cortical resorption prior to day 14 HLU,[120] and a longitudinal assessment of tibial trabecular bone density using a PWB model saw vBMD loss by day seven, with the degree of change being load-dependent.[72]

Of interest, of the 17 studies incorporating irradiation as an experimental condition, the majority demonstrated exacerbation of the observed HLU effects on bone loss; whether this was an additive or synergistic effect is unclear.[186] However, all but one of the scored publications used acute exposures of irradiation; the single publication that used chronically-administered, low dose rate irradiation demonstrated no exacerbation of HLU effects following concurrent exposure,[187] suggesting that space-relevant exposures may not play a large role, although further work is needed to confirm this conclusion given the level of uncertainty in the study parameters.

3.2.1.3. *Recovery Kinetics*: With respect to interplanetary exploration, skeletal deficits may play a critical role in the postflight period. However, similar to the induction time line, observed patterns of recovery in astronauts demonstrate significant variability, dependent on inter-individual heterogeneity,[188] age,[84] and the choice of analytic approach,[179] *e.g*., comparing results following the use of either DXA or QCT.[154, 188] Recovery of integral BMD has been demonstrated in some astronauts at approximately 1-year postflight,[131] although recovery of cortical and trabecular density, as well as bone strength *per se*, appears to take considerably longer, albeit dependent on the skeletal bone in question.[85, 132, 189] Other studies have suggested persistent decrements[132, 188] with one recent study showing a significant effect of mission duration, likening the effect seen after ~six months of spaceflight to a decade of age-related bone loss.[188] Interestingly, some studies have suggested that recovery during the first year is achieved through a hypertrophic mechanism leading to an increase in bone size and cross-sectional area,[85, 189] whereas Gabel *et al*. indicated that there was greater bone turnover in those astronauts that failed to demonstrate complete recovery.[188] Of note, some have suggested that the ongoing process of age-related bone loss may lead to a downstream resumption of decrements in astronauts in subsequent years, in conjunction with an increase in the potential risk of age-related fractures.[77, 190]

Bed rest studies, whether horizontal[93] or HDT,[95, 140] have identified a period of continued bone loss following reambulation, with a nadir at around 15—20 days.[92, 93, 168] This progression has been shown in some studies to be accompanied by increased levels of bone resorption markers,[93, 97] possibly in response to microdamage induced by gravitational reloading, although this phenomenon has not been seen consistently.[138, 191] As noted in astronauts, differential loss and recovery rates have been seen between cortical versus trabecular bone, although in contrast, some studies have shown the greatest total loss and slowest recovery to be in the cortical compartment.[92, 93] Indeed, Cervinka *et al*. suggested that the measurement of absolute cortical bone loss exceeding that of trabecular bone during shorter bed rest studies may be due to the accelerated cortical bone loss seen in the immediate (2—3 weeks) reambulation period.[168] Overall, in a similar trend to that seen in astronauts, recovery of most bone parameters appears to take longer than the induction,[76] estimated to be 2—3 times longer than the original bed rest period.[92, 93]

As noted previously, few of the scored space rodent studies performed analyses beyond the immediate postflight period. Of those, in early rat spaceflight studies, Jee *et al*. demonstrated recovery of trabecular bone volume by day 29,[107] and a similarly rapid return to baseline was seen with respect to periosteal bone formation.[108] Using a HLU 6-month old rat model, Shirazi-Fard *et al*. undertook a serial examination of bone recovery kinetics over 84 days (3x the period of HLU);[147] they saw site-specific recovery in the proximal tibial metaphysis (PTM), recapitulating the heterogeneous dynamics of recovery seen in astronauts.[189] However, although bone mass recovered over a period twice as long as that of unloading, bone density and cortical thickness failed to return to pre-unloading levels within a period 3x unloading, supporting similar findings from other animal and astronaut studies.[77, 192]

3.2.1.4. *Potential Mechanisms*: Identifying the specific mechanisms that underlie spaceflight-induced bone loss is critical in order to develop countermeasures. During early Apollo missions, a significant increase in fecal and urinary calcium loss was observed, even after short (12 day) flights;[193] later studies, conducted during the Shuttle-Mir period, indicated that intestinal absorption of calcium also was significantly decreased during flight.[86, 194] Subsequently, urinary calcium levels have been shown to increase rapidly within the first few weeks of flight, progressing over time, albeit at a slower rate.[84, 195, 196] However, absolute figures have been difficult to determine due to inherent individual variability in calcium excretion, combined with small group

sizes and limited sampling time points.[197] In addition, many of the more recent astronaut studies examining urinary calcium have been performed in the context of assessing the impact of nutritional,[84, 198] exercise,[199, 200] or pharmaceutical interventions[199-201] used as countermeasures against bone loss and/or reducing renal stone risk, obscuring changes in absolute baseline levels.

Human HDT bed rest studies also have demonstrated a rapid and sustained increase in urinary calcium excretion during bed rest,[94, 134, 141] although to a lesser degree than that seen in astronauts.[135] It again appears likely that the finer kinetics of calcium excretion in the bed rest model have been obscured by limited study lengths, few interim analyses, and the inclusion of countermeasure arms. Nonetheless, some investigators have used the HDT bed rest model to address whether calcium levels play a role in space-induced bone loss. For example, a number of bed rest studies have indicated that dietary supplements directly manipulating calcium balance (*e.g*. additional calcium, vitamin D) have failed to improve bone resorption/formation;[202, 203] this finding was verified in space, albeit in a limited number of small astronaut studies.[204] Interestingly, a 4-week HDT bed rest study performed by Arnaud *et al*. identified a potential link between calcium excretion and a high salt diet;[205] a similar and significant association between sodium intake and urinary calcium was subsequently demonstrated in astronauts.[84]

We are unaware of any space rodent studies that have directly addressed calcium balance. Of the few scored ground-based studies performed in this area, one detailed HLU study looked at metabolic changes induced by different diets, and demonstrated a differential in urinary calcium levels dependent on diet; tellingly, this outcome appeared to be unaffected by HLU and unrelated to bone loss.[123] Another group studying calcium balance in HLU rats showed a reduction in calcium absorption associated with high sodium intake[122] and later suggested that increased calcium secretion was not due to urinary excretion, but increased fecal calcium levels alone,[206] contrary to that seen in humans.[205]

Evolving insights into the mechanisms of normal mechanotransduction and the role(s) played by various cell types in the bone microenvironment[182] have led some investigators to point to the development of a critical imbalance ("decoupling") in normal osteoblast and osteoclast homeostasis driven by the absence of gravitational loading.[207, 208] This imbalance has been hypothesized to be the primary mechanism leading to increased bone resorption,[207, 209] since it provides an explanation for the differential changes seen in bone resorption versus bone formation markers in astronauts, *i.e*. an increase in resorptive markers versus decrease/plateau in formation

markers, respectively.[85, 86, 210] Although the specific mechanism(s) underlying the imbalance remains a matter for conjecture, some reviews have pointed to the differences in physical response to weightlessness seen in osteoblasts versus osteoclasts,[182, 207] suggesting that osteoblasts undergo significant physical damage under microgravity conditions, whereas osteoclasts are able to maintain their function. However, it is worth noting that the description of overt osteoblast effects appears to be based on a limited number of spaceflight *in vitro* studies.[209, 211] Furthermore, although the majority of human HDT bed rest studies have demonstrated similar rapid (within days) increases in markers of bone resorption, little to no change has been seen in bone formation markers across the time line of bed rest,[134, 135, 184, 212] with at least one study failing to see significant changes in either parameter.[139]

Another proffered explanation for space-induced bone loss has focused on osteocytes alone, identified as a central regulatory cell in the maintenance of bone homeostasis.[207, 213] *in vitro* studies have suggested that osteocytes sense mechanical loading through fluid shifts,[214-216] subsequently inducing a response in the bone microenvironment through the release of various regulatory signals, *e.g.* PGE2, NO and ATPs.[217] A role for fluid shifts in space-related bone loss also has been related to the observed muscle atrophy (**see 3.2.2.**), reinforcing the intimate connectivity that exists between bone and muscle.[218] However, although there is evidence of osteocyte loss from both murine spaceflight[219] and HLU[220] studies, and *in vitro* space studies have demonstrated impairment of osteocyte differentiation,[221] it is not clear that astronaut studies *per se* have provided confirmatory evidence for this mechanism. Nonetheless, serum sclerostin, a regulatory signal released by osteocytes that inhibits bone formation, has been shown to increase during bed rest,[185, 222, 223] providing additional support for disrupted homeostasis playing a role in the observed bone loss.

Surveying the scored animal studies, although limited in number, rodent spaceflight studies also have demonstrated osteoblast-osteoclast-osteocyte dysregulation. Some early investigations generated mixed results,[224] with rat studies suggesting that, contrary to astronaut findings, there was increased bone formation, whereas resorption remained stable.[107, 225, 226] This observation may have been the result of rats only achieving skeletal maturity towards the end of their lifespan, so that the observed bone loss reflects growth failure rather than homeostatic disruption.[227] However, more recent studies have demonstrated patterns of increased osteoclast-mediated bone resorption in the murine space model,[104, 170] as well as decreased osteoblast-mediated bone formation.[106, 170]

In addition, one study, looking at murine microstructure of trabecular and cortical bone, demonstrated differential changes between weightbearing and non-weightbearing bones associated with compromised osteocyte lacunae, suggesting significant osteocyte death.[219] Furthermore, as noted previously, spaceflight *in vitro* studies of murine osteoblasts versus osteoclasts have been used as evidence that osteoclast histomorphometry and function are less affected by weightless conditions than osteoblasts.[209, 228]

Although the majority of ground-based rodent studies have focused on identifying subcellular and/or molecular changes, some have addressed broader mechanisms. Yang *et al*. demonstrated an increase in osteoclast numbers (and a corresponding decrease in osteoblasts) within seven days of starting HLU;[124] exacerbated osteoclastic bone resorption also has been seen in studies combining HLU with irradiation.[173, 175] Interestingly, one combined HLU and radiation study suggested that, although both irradiation and HLU independently induced an increase in osteoclast numbers, HLU alone affected osteoblast function.[177] Alternatively, Steczina *et al*. posited that the reduced osteoblast function may be due to an HLU effect on osteoblastogenesis,[186] whereas Macias *et al*. indicated that this specific process was inhibited by irradiation alone.[111] Several rat studies also have demonstrated a decrease in osteoblast activity whilst maintaining osteoclast function,[72, 229] suggesting a species-dependent difference. The potential for osteocytes to play a critical role in microenvironmental imbalance also has been recognized in ground-based models.[60, 120]

3.2.1.5. *Other Skeletal Issues*: Intriguingly, although there has been an overall focus on physiologic mechanisms underlying bone loss in the weight-bearing limbs, some astronaut studies have indicated a simultaneous increase (+2.2%) in cranial bone density.[180, 230] This phenomenon also has been seen in at least one space animal study,[100] potentially offering insight into mechanistic links between fluid shifts and changes in bone density. In addition, animal space studies have demonstrated degradation of weight-bearing articular cartilage, with no effect on minimally-loaded sternal fibrocartilage;[103] such degradation is associated with an arthritic phenotype,[231] a condition also seen in HLU models.[232] Despite these data, we identified few astronaut studies that examined cartilage changes, other than indirectly assessing intervertebral disc integrity and back pain.[161] However, some bed rest studies have pursued this issue and demonstrated articular

cartilage thinning in weight-bearing joints, *e.g*. the knee, evident seven weeks after initializing partial-weight bearing.[233]

3.2.2. Muscle Effects

3.2.2.1. *Muscle-Associated Outcomes*: The remodeling observed in astronaut skeletal musculature under microgravitational conditions results in loss of muscle volume and strength,[234, 235] fatigue resistance[236] (particularly appreciated during the recovery period[237, 238]), and motor performance.[239] During the Skylab missions in the 1970s, muscle adaptation was monitored and significant loss of volume from the lower limbs was seen within the first few days in space.[239] The potential muscle atrophy quickly led to the introduction of various exercise technologies and protocols[240, 241] as part of astronauts' health maintenance programs, and significant improvements have been observed subsequently.[128] Worth noting is that varying levels of participation in available exercise protocols by individual astronauts[129, 242, 243] correlates to heterogeneity in outcome metrics, confirming the utility of such countermeasures. For example, Greenisen *et al*. demonstrated greater decrements in astronauts that had not exercised during short-flight Shuttle missions, although the observed benefits of exercise varied dependent on the targeted muscle.[244]

Although the introduction of increasingly aggressive exercise programs, including the incorporation of resistance exercise, have led to progressive improvements in outcomes compared to those seen during Skylab and Apollo missions,[127, 235, 245] nonetheless loss of volume from muscles involved in posture maintenance and stability is still evident, albeit variable dependent on the muscle. For example, in the lower limbs, a decline in volume is greater in the calf versus thigh muscles.[235, 245] In the calf alone, the soleus muscle undergoes greater loss than the gastrocnemius,[235, 246] with both slow twitch (type I) fibers and fast twitch (type II) being affected,[247, 248] whereas in the thigh, hamstrings lose greater volume than the quadriceps.[245, 249] Interestingly, at least one study has suggested that the greatest relative deficit appears in the back muscles; after 23 weeks in space, 20% loss of volume was recorded in the intrinsic back muscles versus 12—16% loss in quadriceps/hamstrings.[249] It was proposed that such changes may contribute to the lower back pain[83] and increased risk of disc herniation[15] experienced both during and postflight by some astronauts.

However, assessing change in muscle volume is an error-prone measurement, susceptible to fluid shifts[250] and other factors.[235] Since this process has been mostly (~80%) ascribed to muscle

atrophy, quantitative analyses specific to assessing atrophy *per se* have been performed.[251] The extent of atrophy has been frequently determined through analysis of muscle fiber cross-sectional area (CSA),[126, 129, 161, 238, 252] and/or intramuscular fatty infiltration,[252, 253] the latter observed as attenuation on X-ray imaging.[129] Using data from muscle biopsies, Greenisen *et al*. described a 15% loss in the CSA of type I fibers versus 22% CSA loss in type II fibers; this was accompanied by a change in fiber distribution, with an increase in the percentage of type II fibers versus a loss of type I.[244] A differential loss of fiber type was supported by the work of both Edgerton *et al*.[238] and Widrick *et al*.,[254] and a similar shift in slow-to-fast myofibers was observed in some bed rest studies.[255] The more qualitative parameters of muscle endurance,[235] performance[256] and strength[128] also have been assessed in astronauts; in one study, ~163 days of spaceflight resulted in 8—17% loss of isokinetic strength, with a non-statistical (improvement) effect due to changes in resistance exercise technologies.[127] Of note, in this particular study, there were indications of greater loss in female astronauts on recovery day five, although, overall, there did not appear to be statistically significant differences dependent on gender.[127]

For the specific purpose of assessing skeletal muscle changes following unloading, in addition to the horizontal/HDT bed rest and dry immersion models used in bone studies, other simulation models have been used, including limb immobilization[257, 258] and unilateral lower limb suspension.[259, 260] For example, using unilateral limb immobilization, Deschenes *et al*. demonstrated greater decrements in muscle power and performance of females following seven days of disuse compared to males in the absence of a differential change in muscle mass;[88, 89] this effect appeared to be related to parallel reductions in the neural activation of maximally contracting muscles.[88] However, irrespective of the model, human studies assessing muscle changes as a result of disuse have demonstrated similar findings to those seen in astronauts, including the susceptibility of postural muscles to volume loss, with little to no effect on upper body musculature,[261] and loss of strength relative to muscle area/volume during recovery.[262] In addition, increased deposition of intramuscular adipose tissue has been seen during bed rest,[263] although not during spaceflight,[253] and the findings with respect to loss of muscle fatigue resistance are similarly equivocal.[264]

Bed rest induces similar patterns of loss in muscle volume from the lower weight-bearing limbs as seen in astronauts, with the greatest reductions seen in calf and thigh muscle volume, together with an associated decline in strength.[261, 265-267] As recognized in astronaut studies,[161, 268]

evidence of lumbar spine muscle atrophy has been seen during prolonged bed rest in association with lower back pain.[269] In addition, measurement of muscle CSA has suggested similar patterns of atrophic induction in ground-based human disuse models to those seen in space,[270-272] although several studies with evidence of decreased muscle CSA failed to see corresponding X-ray attenuation, instead suggesting a loss of intramuscular lipid stores.[273, 274] Certainly, integrated aerobic and resistance exercise has been shown to mitigate loss of muscle power and CSA reduction in 14 days horizontal[275]/60 days HDT,[267, 276] correlating to the benefits seen in astronauts. Nonetheless, differences in outcomes have been observed between the two models; for example, Hides *et al*., in a 14-day bed rest study, demonstrated an increase in trunk flexor muscle CSA, possibly reflecting overactivity and/or muscle shortening, affecting the risk for lower back pain.[277] In addition, although some bed rest studies have seen atrophy and strength loss in specific hip-related musculature,[278, 279] this particular physical area has received little focus in astronauts.[239]

Few of the scored studies in flown rats or mice addressed muscle changes. Since qualitative functions, such as muscle performance and fatigue, are difficult to assess in animal models, endpoints have focused predominantly on physiological or pathological alterations and, in general, have demonstrated parallel responses to those seen in humans. One early rat spaceflight study showed more extensive muscle loss from the soleus muscle (-23%) versus the extensor digitorum longus (EDL) muscle (-11%) following seven days of spaceflight;[280] the EDL is frequently used to assess the response of a non-load bearing muscle. In a separate study, although 14 days of flight resulted in equivalent muscle mass loss from the gastrocnemius (-8.9%) versus EDL (-8.5%) muscles, measurement of fiber CSA showed a ~25% versus ~13% reduction, respectively, supporting a differential level of response between the two muscle types.[281] Radugina *et al*. demonstrated significant atrophy of the mouse quadriceps after 30 days of flight compared to either vivarium and ground controls, assessed histomorphologically using parameters such as reduced muscle fiber size.[282] Importantly, Ishihara *et al*. showed a lack of response in the triceps of mouse forelimb after 13 days of flight,[283] supporting a preferential muscle loss from the hind limbs despite a quadrupedal conformation. However, in contrast to astronaut studies, a preferential loss of type I versus type II fibers from the rat soleus and medial gastrocnemius has been seen,[284] and others failed to see a shift in slow-to-fast (or even fast-to-faster) myofibers following a 14-day flight.[281]

Ground-based rodent studies have demonstrated a similar overlap with the effects seen following human bed rest, *etc*. Using a mouse model, Morris *et al*. demonstrated time-dependent

loss of muscle mass over 14 days of HLU,[143] with greater loss from the soleus versus the gastrocnemius. Interestingly, Arbogast *et al*. performed an *ex vivo* analysis in isolated soleus muscles and showed a faster onset of fatigue in muscles from the unloaded group compared to controls.[117] A number of investigators have demonstrated similar decrements in the rodent gastrocnemius after 14 days HLU, assessed by a reduction in muscle weight[152, 285, 286] and declines in protein synthesis.[151, 152] Worth noting is that Thomason *et al*. demonstrated abrogation of soleus atrophy in a rat model by providing activity during the course of HLU,[287] a potential equivalent to the use of exercise as a countermeasure in humans. In addition, it is important to highlight a recent study performed by Mortreux *et al*., in which the group systematically determined muscle adaptation in male versus female rats over 14 days HLU.[116] They demonstrated differential muscle-specific differences between the genders; for example, both sexes demonstrated equivalent loss from the gastrocnemius and EDL muscles, but greater loss from the soleus and tibialis anterior in the males. However, overall, the data regarding gender-based differences is mixed: Deschenes and Leathrum demonstrated greater unloading-related atrophy in the soleus muscle of male versus female rats;[81] Rosa-Caldwell *et al*. showed higher induction of atrophic markers in females during the early period (24 hrs) of disuse,[115] whereas an earlier study had shown no sex-related differential;[288] Mortreux *et al*. demonstrated equivalent reductions in muscle CSA in both sexes, the mechanism of loss appeared different, with males demonstrating a reduction in type 1 CSA versus a reduction in type 2/hybrid myofiber CSA in the females.[116] Overall, it appears that the temporal progression of muscle adaptation is a complex response, with a potential for muscle-specific differences between the two sexes,[115] meriting more in-depth study to determine risks based on gender-specific differences in muscle response.

3.2.2.2. *Time Line of Progression*: As noted previously, the majority of analyses conducted in astronauts are performed on samples taken pre- versus postflight, rather than during weightlessness.[239] Furthermore, the delay in collecting postflight data, often measured in days, if not weeks, after return, likely further confounds precise determination of temporal changes. This may be critical given the profound pathological changes that are seen when comparing inflight to immediate postflight samples.[289] Nonetheless, it does appear that muscle volume loss is flight-duration dependent, with one study determining the rate of loss at 0.62% to 1.04% per day in the initial few weeks.[243] Other studies have indicated that muscle volume loss is non-linear over time,

with ~5% loss over the first week of spaceflight,[245] attenuating to 1.6% per week over the second and third weeks of flight,[249] but reaching a steady state after ~four months in space, resulting in an average loss across a long-term (~6-month) mission of 0.5% per week, albeit with considerable individual variability.[249]

The overall relative loss of muscle volume appears lower following human ground-state disuse than that seen during spaceflight, by as much as a factor of two in some muscles, with similar levels of individual heterogeneity.[243, 290] Interestingly, the National Aeronautics and Space Administration Bed Rest Study, conducted over 70 days of bed rest, demonstrated a linear rate of muscle atrophy,[291] whereas Miokovic *et al*. showed differential rates of atrophy during bed rest, not only between different muscles, but also, in some cases, along a single muscle.[96] Indeed, in most studies, a rapid onset of symptoms has been demonstrated followed by a plateau; a recent multi-study review, looking at 75 human bed rest studies, showed that, without exercise, moderate changes in a range of muscle parameters occur within 7—14 days of unloading, with maximal effects seen at around day 30.[292] As seen in astronauts,[244] muscle strength appears to decline faster than atrophy, with the ratio of muscle strength:atrophy as a function of bed rest duration being 4.2 on day five, falling to 2.4 by day 14, before plateauing at 1.9 by ~day 35 of bed rest.[251] As might be predicted, direct comparisons between spaceflight and human ground-based models are confounded by experimental limitations, making the prevalence and progression of muscle adaptation during immobilization relatively unknown, particularly over the long-term.[257] Furthermore, discrepancies between studies also might reflect relative levels of precision, *i.e*. the relative ability to identify nonlinearities, and it is likely that modeling resulting in linear responses may be due to inherently large uncertainties.

Turning to the animal models, older reviews[293] have suggested a similar non-linear pattern of muscle mass loss from the hind limbs of rats to that seen in humans flown in space, with strong support for a rapid and significant atrophy (-37%) observable within the first week of flight,[284] although the kinetics of any subsequent pattern of atrophy are less clear. Loss also appears rapid over the first three days in ground-based HLU models, with a more gradual rate of decline reaching a quasiplateau of 40—50% loss at day ten,[65, 117, 125] followed by a steady, albeit considerably lower rate of, decline.[294, 295] Unfortunately, the vast majority of rodent studies performed during microgravitational conditions, whether real-time or simulated, have been limited to ≤one month. On the positive side, and as noted in the NASA Evidence Report HRP-47072,[239] rodent

musculature is pathologically similar to humans in terms of its fiber profile and response to environmental stimuli, allowing measured comparisons to be drawn. However, the observed adaptive changes in rodents occur over a much shorter period of time; therefore, although it has been generally stated that the qualitative pattern of response is similar between humans and rodents,[296, 297] with a preferential loss of contractile proteins over others,[284] care must be taken when correlating and/or extrapolating from one species to another given the limited, but differential, comparative adaptive responses seen, for example, with respect to fiber type and extent in rats (see Figure 1 in reference [248]).

3.2.2.3. *Recovery Kinetics*: Early astronaut studies indicated that volume loss, even after short missions, persisted in some muscles for greater than two weeks following return.[245] Persistence was less evident in a small, but methodical, analysis conducted by Greenisen *et al*. who demonstrated a similar level of loss in strength on landing, but with recovery by day seven.[244] This has led to suggestions that the immediately observed decrements may be partially due to muscle damage from gravitational reloading,[249] as suggested by animal studies.[289] However, given evidence of muscle damage persisting for 30—80 days of landing after longer-term spaceflights (4—6 months),[249] the space environment *per se* also plays a significant role.

Following 90 days of HDT bed rest, Rittweger *et al*. demonstrated a rapid, but partial, recovery of muscle CSA, irrespective of any countermeasure, followed by a gradual return to baseline with a potential overshoot above baseline at reambulation day ~100;[97] muscle power recovered at reambulation day ~150.[298] In contrast, investigators demonstrated recovery in volume and strength of all muscles by reambulation day 90;[299, 300] although an overshoot (hypertrophy) was seen by some, it was only observed in non-active participants.[299] Scott *et al*. demonstrated faster rates of recovery in those muscles that showed the greatest decline in muscle CSA,[291] however, recovery in this study was only tracked for ten days of reambulation. Although not necessarily related to the level of decline, Miokovic *et al*. demonstrated differential levels of muscle volume recovery, with some returning to baseline between 14 and 90 days following re-ambulation, whilst others, such as the lateral gastrocnemius, continuing to show significant decrement (-30—45%) at 90 days.[96]

As noted previously, few of the scored rodent studies, space or ground-based, examined time points beyond the immediate recovery period. Krause *et al*. demonstrated loss of

gastrocnemius weight after 14 days of HLU, with an associated decline in protein synthesis.[151] Muscle weight was restored within 14 days of reambulation, although additional loading, previously shown to improve bone recovery, had an adverse effect on the muscle.

3.2.2.4. *Potential Mechanisms*: The mechanisms underlying muscle changes seen in astronauts have focused mainly on the induction of atrophy.[248] Disuse atrophy is characterized by decreases in protein synthesis,[301] together with increases in protein degradation rates;[302, 303] both have been observed in astronauts,[304] bed rest patients,[305] and HLU mice,[58] and may account for much of the rapid loss of muscle protein seen during microgravity conditions.[306] Interestingly, some have suggested that the greatest levels of atrophy occur in those crew members with the largest preflight fibers;[238, 307] similarly, one bed rest study indicated that the degree of atrophy related not only to the temporal duration of unloading, but also to pre-unloading muscle size.[308] Unsurprisingly, many of the scored animal studies have focused on the induction of atrophy, with the ability to perform more detailed anatomical examinations leading to greater scrutiny of changes at the cellular level. As noted previously, early flight studies of rats demonstrated a change in distribution of fibers associated with a slow to fast transformation[309] that resulted in a decrease in the percentage of type I fibers versus an increase in type II,[280] also seen in mice.[310] However, results have shown considerable variability with, for example, Kraemer *et al*. showing a predominant effect in fast twitch fibers,[311] whereas Ohira *et al*. graded atrophic effects as slow extensors > fast extensors > fast flexors, with reductions varying with respect to the predominant fiber of the observed muscle.[312] Whether the current advances in -omics will offer further insights into the mechanisms underlying space-related muscle atrophy will likely be the focus of future studies.[310, 313, 314]

However, although ~80% of muscle strength loss has been attributed to atrophy, the remaining deficit is likely due to a number of different processes, including single fiber mechanical properties and architecture, neuromuscular damage and supraspinal changes.[251, 264] Indeed, recent reports have highlighted the lack of attention paid to other potential factors that may play a role in atrophy induction itself, including radiation, nutritional status, hormonal disruption, *etc*.[246, 315] Interestingly, at least two interventional bed rest studies, designed to counter muscle atrophy through the administration of nutritional supplements,[316, 317] resulted in exacerbation of bone loss, not only emphasizing the role of nutrition in muscle atrophy, but also highlighting the interdependence of muscle and bone. This connectivity has been reinforced by rodent studies,

performed in space and on the ground, showing that myostatin, a member of the transforming growth factor-ß superfamily that regulates skeletal muscle mass, is upregulated during microgravitational conditions and its inhibition appears to protect against loss of both muscle and bone.[218, 318, 319]

4. Vascular (Cardio-/Cerebrovascular) Outcomes

Data from the A-bomb survivors[320] and radiation therapy patients[321, 322] has brought attention to the potential risk of long-term vascular effects in astronauts as a result of exposure to space radiation, *i.e.*, from galactic cosmic radiation (GCR) and/or solar particle events (SPEs).[323-325] Tellingly, cardiovascular-related symptoms, such as tachycardia and orthostatic intolerance, have been observed in astronauts and cosmonauts, both during flight and immediately following landing,[326, 327] in addition to frequent reports of headaches experienced inflight.[328] Such symptoms led to a large series of national and international studies investigating whether spaceflight imposed a risk of vascular disease on astronauts, with specific concerns that microgravity and space radiation may act synergistically on the cardiovascular system; this subject has been covered extensively elsewhere.[324, 329-331] In addition, a range of neurovascular symptoms, some of which will be addressed in **5. Central Nervous System (CNS) Outcomes**, are also affected by alterations in the cerebral hemodynamics experienced under microgravity, providing links between vascular effects and other endpoints, *e.g.* musculoskeletal changes.[61, 332, 333] This section will focus on generalized vascular-related symptoms, such as changes in plasma volume, cardiac output, blood pressure, arterial/venous stiffness and resistance, and cerebrovascular blood flow as endpoints.

4.1. Publications Overview

4.1.1. *Astronaut Category* (**Table 5**)

- Although gravitational changes are known to affect endothelial cells[334] and loss of gravity is believed to initiate "ageing-like" deconditioning on the cardiovascular system,[324] only 3/20 of the astronaut studies assessed in this category were found to focus on physical vascular changes *per se*.[335-337]
- Clear mechanistic differences have been recognized in the appearance, treatment and outcomes of cardiovascular[338, 339] and cerebrovascular[340, 341] diseases between men and women, however only one of the publications scored in this category addressed gender as a factor,[337] even when there were sufficient numbers of both sex in the cohort.[342]
- Eleven of the studies[335-337, 343-350] looked at inflight changes although the number of sampling times were insufficient to fully determine effect progression, and only two of the studies included time points beyond the immediate recovery period,[336, 342] limiting the ability to correlate acute postflight changes to any long-term prognosis.

4.1.2. *Ground-Based Human Category* (**Table 6**)

- Although there is some evidence of a direct effect of hypercapnia on both cardiovascular[351] and peripheral vessels,[352] we are unaware of any human ground-based studies addressing this issue, although four of the 36 studies scored in this section addressed its effects on the cerebrovasculature.[353-356]
- Similar to observations in the musculoskeletal section, only 15/36 of the scored studies involved female subjects, with none addressing gender as a factor (three studies used females only[357-359]). Two of the scored studies involved age-appropriate adults[353, 354] and, despite the obvious relationship between physical fitness and vascular health, as well as the inherent physical status of the comparative astronaut cohort, only ten of the scored studies described the fitness level of the participants.
- No time points were assessed beyond the immediate (days 1—14) reambulation period, although most addressed interim time points during immobilization.

4.1.3. *Animals in Space Category* (**Table 7**)

- All studies scored in this category used rodent models, with a ratio of 3:9 rat to mouse subjects; all but one of the murine studies used C57BL/6. Similar to the studies scored in the musculoskeletal section, four of the studies involved juvenile animals,[360-363] with none using appropriately aged animals (≤six months). However, unlike the musculoskeletal system, the hearts of both mouse and human are, for most parameters, fully developed shortly after birth,[364] so that age might not represent a directly contributing physiological factor. 6/12 of the scored publications used females, but none compared data between sexes.
- Some of the studies were performed using a full complement of control groups, including age-matched cohorts maintained under replica housing conditions to those in space (*e.g.* AEMs), as well as a matched vivarium cohort.
- Interestingly, although vascular effects, and cardiovascular deficits in particular, take months if not years to develop, none of the publications scored in this category investigated time points beyond the first day of recovery and no studies involved animals undergoing spaceflight longer than ~30 days, with only three looking at samples captured under weightless conditions.[365-367]

4.1.4. *Ground-Based Animal Category* (**Table 8**)

- All studies considered in this category used rodent models, with a ratio of 17:4 rat to mouse subjects, suggesting an investigator preference for using the larger species when researchers are not constrained by payload limitations. The majority of the rat studies utilized the Sprague-Dawley strain, whilst the murine studies were conducted in C57BL/6 only.
- As noted earlier, there are data suggesting a potential risk of long-term cardiovascular effects as a result of astronaut exposure to radiation, *e.g.*, galactic cosmic radiation (GCR),[323, 324] however, only two of the 21 ground-based studies considered in this category included radiation in their experimental design.[150, 368] The majority of the studies made use of individual housing, a significant factor in the cardiovascular studies given the potential interaction between social isolation and hindlimb unloading on hemodynamic parameters.[369] One study[368] used females alone and none of the scored publications examined gender as a factor. Again, this reflects a relative paucity of gender studies in the field, although some direct comparisons between males and females have been performed in other cardiovascular studies, with gender-based differences being seen.[370] Encouragingly, 8/21 of the studies used age-appropriate animals, with only four using juveniles; the majority of the remainder used young adults.
- Only six studies made use of interim time points during unloading,[371-375] and only one study[368] investigated time points beyond the first hours/day of recovery.

Table 5: Highest scoring publications in the astronaut vascular category.

ASTRONAUT: CARDIOVASCULAR	Environment parameters	Astronaut characteristics	Science / time points	Score	Main Scientific Findings
Short flight (≤30 days)					
Fritsch-Yelle JM *et al*. 1996[345]	9	3	6	18	5—10 days flight: Basic parameters measured pre-, mid- and postflight. Heart rate, arterial pressure and cardiac rhythm disturbances decreased during flight.
Leach CS *et al*. 1996[346]	9	3	5	17	9 or 14 days flight: Plasma volume (PV†) fell within 21 hrs of launch and persisted until after landing, with fluid shifting from extra- to intracellular compartment
Meck JV *et al*. 2001[376]	9	3	5	17	7—9 days flight: Compared to long-flight; changes in HR†† and PV similar between groups. Long-term flight increased risk of orthostatic intolerance
Summers RL *et al*. 2007[377]	9	3	5	17	9—16 days flight: Compared to ground controls, echocardiography showed reduction in LVM†††, with rapid recovery (3 days) postflight
Bungo MW *et al*. 1989[378]	9	3	4	16	5—8 days flight: Echocardiography indicated significant changes in heart volume affecting left ventricular function at R0, with some persistence at R7—14 days
Perhonen MA *et al*. 2001[379]	9	3	3	15	10 days flight: Pre- vs. postflight MRI†††† of astronauts (vs. bed rest) used to assess cardiac atrophy showed decreased cardiac mass
Long flight (≥4 months)					
Fu Q *et al*. 2019[343]	9	4	5	18	~6 months flight: Comparisons of pre- and postflight orthostatic tolerance demonstrated benefits of counter-measures
Arbeille P *et al*. 2016[348]	9	4	5	18	~6 months flight: Echocardiography showed increased IMT* in both carotid and femoral arteries within 15 days of flight, remaining elevated throughout flight
Lee SM *et al*. 2020[335]	9	3	5	17	~190 days flight: Focus on carotid and brachial arteries. Markers of OS** and inflammation increased during flight; normalized by R7 days.
Marshall-Goebel K *et al*. 2019[336]	9	3	5	17	~210 days flight: Focus on IJV***. Stagnant and/or retrograde blood flow observed – risk of thrombosis.

Mulavara AP *et al*. 2018[256]	9	4	4	17	~159 days flight (compared to bed rest+/-exercise). Significant change in HR from prone to standing, but no change in BP****
Hughson RL *et al.* 2016[337]	9	5	3	17	146—193 days flight: ECG pre-, during and postflight: sex-dependent differential changes in insulin resistance and arterial stiffness
Ade CJ *et al*. 2017[342]	9	2	5	16	Longitudinal Study of Astronaut Health. No association with long-term risk of cardiovascular disease
Ade CJ *et al*. 2017[380]	9	4	3	16	~170 days flight: Long-duration spaceflight reduced maximal oxygen uptake, affecting both diffusive and convective O_2 transport
CEREBROVASCULAR					
Short flight (≤30 days)					
Iwasaki K-I *et al*. 2007[344]	9	3	6	18	16 days flight: Both static and dynamic cerebral autoregulation was preserved (or even enhanced)
Long flight (≥4 months)					
Zuj KA *et al*. 2012[381]	9	3	2	14	~150 days flight: Postflight impairment of cerebral autoregulation and CO_2 reactivity

† PV: plasma volume
†† HR: heart rate
††† LVM: left ventricular mass
†††† MRI: magnetic resonance imaging
* IMT: intima-medial thickness
** OS: oxidative stress
*** IJV: internal jugular vein
**** BP: blood pressure

Table 6: Highest scoring publications in the human immobilization vascular category.

HUMAN HDT STUDIES: CARDIOVASCULAR	Environment parameters	Astronaut characteristics	Science / time points	Score	Main Scientific Findings
Short-term (<10 days HDT)					
Convertino VA *et al*. 1994[382]	3	2	3	8	7 days HDT led to peripheral vasoconstriction, potentially related to hypovolemia
Shiraishi M *et al*. 2002[383]	2	2	4	8	Comparison of 30 minutes wet immersion to 30 minutes HDT showed similar cardiovascular effects
Medium-term (10—<50 days HDT)					
Amirova L *et al*. 2020[384]	3	2	7	12	Cardiovascular changes across test period were comparable between 21 days HDT and 3 days dry immersion
Borovik AS *et al*. 2020[385]	2	2	6	10	21 days dry immersion: Strong increase in heart rate and reduction in stroke volume; possible adaptation between days 14—19 as assessed by lower effect on BP†
Ishizaki Y *et al*. 2004[386]	3	2	4	10	20 days HDT showed changes in inferior vena cava dynamics related to orthostatic intolerance
Palombo C *et al*. 2015[387]	3	2	3	8	5 weeks HDT: Common carotid and common femoral arteries showed differential responses to HDT
Stenger MB *et al*. 2012[388]	3	3	2	8	21 days HDT induced significantly higher orthostatic intolerance, decrease in plasma volume; no effect on cardiac function
Sun X-Q *et al*. 2003[389]	3	2	3	8	21 days HDT: higher orthostatic intolerance by day 10 HDT; cardiac output decreased by day 3
Long-term (≥50 days HDT)					
Maggioni MA *et al*. 2018[390]	2	3	7	12	60 days HDT reduced autonomic regulation which persisted beyond R10. Mitigated by exercise
Xu D *et al*. 2020[391]	3	2	6	11	60 days HDT affected both cardiovascular and baroreflex mechanisms; possible effects on orthostatic tolerance
Meck JV *et al*. 2009[392]	3	3	5	11	60—90 days HDT – standardization of HDT protocol for NASA assessment

Platts SH *et al*. 2009[393]	3	3	4	10	60—90 days HDT recapitulated multiple cardio-vascular outcomes: hypovolemia, orthostatic intolerance, etc.
Westby CM *et al*. 2016[394]	3	2	5	10	60 days HDT: PV†† significantly reduced; LV††† volume and mass reduced by ~15% and 14%, respectively
Ferretti G *et al*. 2009[395]	2	2	5	9	60 days HDT reduced both cardiovagal and vascular sympathetic regulation
CEREBROVASCULAR					
Short-term (≤10 days HDT)					
Marshall-Goebel K *et al*. 2018[353]	3	3	4	10	29 hrs HDT±CO_2: IJ CSA* increased with increasing angles of head-tilt. Reduction in plasma/blood volume; no effect from CO_2
Kramer LA *et al*. 2017[354]	4	2	3	9	26.5 hrs HDT (12º)±CO_2: MRI: HDT decreased cerebral blood flow, altered cranial anatomy and physiology. CO_2 augmented CSF pulsatility
Pavy-Le Traon A *et al*. 2002[359]	3	2	4	9	Seven days HDT did not affect dynamic of cerebral auto-regulation in females. Suggestion of phases in adaptation: acute - increase in CR** resistance; delayed (~15 days) - return to baseline
Medium-term (10-<50 days HDT)					
Laurie SS *et al*. 2020[355]	4	3	7	14	30 days HDT±CO_2: No changes in cerebrovascular reactivity
Roberts DR *et al*. 2021[356]	4	2	7	13	30 days HDT±CO_2: All participants showed decreased cerebral perfusion. Greatest reduction in SANS vs. non-SANS groups
Lee JK *et al*. 2021[396]	3	2	8	13	30 days HDT: Upward shift of the brain with concomitant intracranial free water redistribution

† BP: blood pressure
†† PV: plasma volume
††† LV: left ventricular
* IJ CSA: internal jugular cross-sectional area
** CR: cerebral vascular index
*** IOP/ICP: intraocular pressure/intracranial pressure

Table 7: Highest scoring publications in the space animal vascular category.

ANIMAL-SPACE STUDIES: CARDIOVASCULAR	Environment parameters	Astronaut characteristics	Science / time points	Score	Main Scientific Findings
Short flight (~30 days)					
Andreev-Andrievskiy A *et al*. 2017[366]	8	2	6	16	30 days in space provided details of changes in BP/HR† measured by implanted telemetry
Ogneva IV *et al*. 2014[397]	8	2	4	14	30 days in space led to elevated stiffness of the cortical cytoskeleton in cardiac myocytes
Stabley JN *et al*. 2012[398]	7	2	4	13	15 days in space reduced vasoconstrictive properties in skeletal muscle vasculature
Behnke BJ *et al*. 2013[360]	7	2	4	13	15 days in space reduced vasoconstrictive properties in mesenteric artery and vein
Ogneva IV *et al*. 2018[365]	7	2	4	13	30 days in space induced mRNA (but not protein) changes in cardiac and lung tissues
Goldstein MA *et al*. 1992[399]	7	1	4	12	14 days in space induced reduction in cardiac muscle CSA††; increase in mitochondrial volume density
Kumar A *et al*. 2021[400]	7	1	3	11	15 days in space induced upregulation of OS†††-related genes in ventricular tissue
CEREBROVASCULAR					
Short flight (~30 days)					
Sofronova SI *et al*. 2015[401]	8	2	4	14	30 days in space induced changes in vasodilation and constriction properties in cerebrovascular vessels
Mao XW *et al*. 2019[362]	8	0	6	14	35 days flight: Increase in retina and retinal endothelial cell apoptosis. Disruption of blood-retinal barrier
Yamasaki M *et al*. 2004[367]	6	1	4	11	16 days flight: Development of aortic nerve and baroreflex system in 9-day old neonates. 50% mortality inflight. Decrease in numbers of unmyelinated aortic fibers; similar to HLU

† BP/HR: blood pressure/heart rate
†† CSA: cross-sectional area
††† OS: oxidative stress

Table 8: Highest scoring publications in the ground-based animal vascular category.

GROUND-BASED ANIMAL STUDIES: CARDIOVASCULAR	Environment parameters	Astronaut characteristics	Science / time points	Score	Main Scientific Findings
Short-term (≤10 days)					
Colleran PN *et al*. 2000[371]	4	4	4	12	Differential changes in skeletal perfusion over time (10 mins, 7, 28 days). Diminished blood flow to hind limb versus increased in forelimbs, head, etc.
Martel E *et al*. 1994[372]	4	2	4	10	24 hrs. HLU: Disruption of the baroreflex control of heart rate seen within 6 hrs suspension and persisted for >1 hr after reambulation
Medium-term (10—30 days)					
Seawright JW *et al*. 2017[368]	5	3	6	14	21 days combined HLU and low-dose γ-radiation (0.04 Gy) induced changes in OS† at early time points. Normalized by 9 months
Ghosh P *et al*. 2016[150]	5	2	4	11	13—16 days HLU reduced vasodilator (not vasoconstrictor) responses; greatest in HLU-^{26}Fe combination.
Colleran PN *et al*. 2000[371]	4	4	3	11	Differential changes in skeletal perfusion over time (10 mins, 7, 28 days). Diminished blood flow to hind limb versus increased in forelimbs, head, *etc*.
Prisby RD *et al*. 2015[402]	4	3	3	10	14 days HLU induced reductions in bone and marrow perfusion associated with reduced endothelial-dependent vasodilation
Zhang LF *et al*. 2008[374]	4	1	4	9	28 days HLU increased BP†† and HR†††; persisted shortly after reambulation
Wilkerson MK *et al*. 1999[62]	4	1	3	8	2-weeks HLU had no effect on splenic or mesenteric resistance artery morphology
Summers SM *et al*. 2009[403]	4	1	3	8	20 days HLU decreased contractile response in aortic thin filaments via p38^{MAPK} pathway
Zhang R *et al*. 2012[404]	4	1	3	8	21 days HLU enhanced maximal contractile response and impaired endothelial-dependent relaxation in basilar and common carotid arteries mediated through OS pathway
Zhang R *et al*. 2009[405]	4	1	3	8	21 days HLU: Differential response to HLU dependent on artery type: involvement of OS

CEREBROVASCULAR					
Short-term (≤10 days)					
Wilkerson MK *et al*. 2002[406]	2	3	3	8	10 minutes vs. 7 days HLU: HR unchanged; MAP* elevated at 10 mins. Total brain blood flow reduced by 48% and 24%. Regional differences in brain blood flow due to increased vascular resistance.
Medium-term (10—30 days)					
Taylor CR *et al*. 2013[363]	2	2	4	8	16 days HLU mice (vs. 13 days spaceflight rats): Differential values for vasoconstrictor response, vascular distensibility and arterial stiffness between space and HLU ground
Wilkerson MK *et al*. 1999[62]	4	1	3	8	14 days HLU: HLU induced changes in cephalic arterial pressure; increases in wall stress caused hypertrophy of basilar artery smooth muscle cells
Wilkerson MK *et al*. 2002[406]	2	3	3	8	28 days HLU: HR unchanged. Total brain blood flow reduced by 27%. Regional differences in brain blood flow due to increased vascular resistance.
Prisby RD *et al*. 2006[407]	2	3	3	8	14 days HLU: Vasoconstrictor response is reduced in middle cerebral arteries of HLU rats through altered NOS** signaling

† OS: oxidative stress
†† BP: blood pressure
††† HR: heart rate
* MAP: mean arterial pressure
** NOS: nitric oxide synthase

4.2. Vascular Microgravity Studies: Data Comparisons Across Models

Early evidence of cardiac remodeling[378, 379] and orthostatic intolerance[408, 409] in astronauts led to a relatively active field of research, in particular comparing data from bed rest studies to those from astronauts to determine underlying biological mechanisms. Indeed, cardiovascular deconditioning has long been recognized as a consequence of bed rest[410] and has been characterized in models of microgravity, including HDT bed rest,[411-413] as well as in alternative techniques, *e.g*. dry and wet immersion.[383, 384] As described by Convertino,[414] compromised cardiovascular performance, the occurrence of cardiac dysrhythmias and atrophy have been considered to be key indicators of risk, requiring analysis and, potentially, the need for countermeasures. Encouragingly, there has been little evidence of vascular-related effects affecting mission performance. With respect to long-term vascular effects, one of the scored astronaut publications, a longitudinal study of a large NASA cohort,[342] showed no apparent risk of long-term cardiovascular disease development. This conclusion was disputed in a later review, albeit citing groups with much smaller group sizes,[415] and a recent study using a matched cohort design, comparing 1514 Cooper Center Longitudinal Study participants to 303 astronauts, showed no increased risk for cardiovascular disease (CVD) mortality relative to spaceflight exposure, although there was evidence of an increase in the total number of CVD events.[416]

Orthostatic intolerance postflight initially presented as a significant challenge, affecting ~25% of astronauts returning after short flight missions,[417] with suggestions of days-long persistence after long-term missions.[418] Its induction has been variously associated with a weakened standing vasoconstrictor response,[419] a reduction in plasma volume,[420] reduced stroke volume,[421] and other factors. Of note, females appear more susceptible to orthostatic intolerance, suggesting a gender-related alternative mechanism of induction.[422] Nonetheless, currently applied countermeasures (*e.g*., exercise in space; saline loading and volume resuscitation on landing) have proven to be highly effective,[343, 418] so that direct model comparison of this specific endpoint has not been considered in this report.

4.2.1. *Vascular-Associated Outcomes*

As described in recent reviews,[7, 329, 423, 424] microgravity results in a range of effects in astronauts that contribute to vascular deficits and cardiac deconditioning: a dramatic shift in the body's fluid distribution,[425] with a loss of gradient from the lower compartments to the upper body;[426] a rapid

increase in plasma protein concentration in parallel with a reduction in total plasma volume;[346] distinct vascular changes including differential increases in vessel wall thickness[348] and arterial wall stiffness,[337] with reductions in arterial[350] and central venous pressure,[427] and reduced vascular resistance.[350] In addition, one study has observed blood flow stasis in the internal jugular vein, indicating a potential risk for thrombosis.[336] With specific respect to the heart alone, an increase in cardiac output of 18—26% has been observed,[350, 428] as well as changes in cardiac muscle mass, particularly of the left ventricle (LV).[377, 379]

A number of overlapping outcomes suggest that the majority of ground-based human models, including parabolic flight, dry immersion, and HDT bed rest, offer a reasonable simulation of space with respect to vascular deconditioning. Demonstrated effects have included a similar fluid shift from lower limbs to upper body,[429, 430] as well as decreases in plasma volume.[346, 384, 388, 393, 394] In addition, intracranial fluid shifts have been seen in 30-day HDT subjects, differentially affecting brain volume,[354, 396] that recapitulate similar shifts seen in astronauts.[431-433] Studies also have shown a decrease in cerebrovascular flow,[354, 434, 435] although with a significant level of heterogeneity,[436] and it has been proposed that these specific observations offer insight into the induction of the spaceflight-related neuro-ocular syndrome (SANS) (**see 5.2.1.2.**). Other observed effects include reduced vascular resistance,[358] reduced central venous pressure,[437] and increased jugular cross-sectional area.[353, 434] With respect to specific cardiovascular changes, subjects in human immobilization studies have demonstrated increased blood pressure,[438] increased cardiac output[383, 429] and reduced stroke volume.[358, 388, 389, 412, 437, 438] Furthermore, decreases in cardiac muscle mass, both left and right ventricle, have been seen following extended periods of bed rest.[357, 379, 394] However, despite the reported similarities to astronaut outcomes, numerous contradictory findings have been described, for example with respect to fluid distribution[439] and observations of decreased/stable cardiac output,[389, 412, 438, 440] increased heart rate,[358, 390, 391, 437, 438, 440, 441] and little to no change in LV mass[440] or cerebrovascular reactivity, with or without the presence of hypercapnia.[355] Interestingly, one bed rest study demonstrated physical vascular remodeling only in the lower limbs.[393] Nonetheless, although levels of effect and the temporal sequence of events do not necessarily coincide with those seen in astronauts, such differences may be wholly or partly dependent on the time of observation during the period of weightlessness and/or immobilization,[412] since a paucity of overlapping time points between the models limits direct comparisons.

Moving to the animal models, it is important to point out that animals, especially rodents, have been considered relatively poor models for investigating space-induced vascular disease.[374, 442] Nonetheless, it has been posited that such models may offer insight into some of the mechanisms underlying more ubiquitous vascular changes, especially at the cellular level.[46] For example, the development of unmyelinated aortic nerve fibers in rat neonates has been shown to be altered by a 16-day flight, indicating potential effects on the baroreceptor reflex.[367] In addition, examination of spaceflight animals by several groups has demonstrated reductions in the mesenteric,[360, 361] gastrocnemius[398] and cerebrovascular[363, 401] vasoconstrictive responses, evident within hours of return and persisting for ~one day postflight.[360] Furthermore, Taylor *et al*. showed stiffening of the posterior communicating arteries and increases in the maximal diameter of cerebral arteries.[363] Taken as a whole, these vascular changes may represent indicators of elevated cerebral perfusion and, again, potential contributors to SANS (**see 5.2.4.**).[363] However, the observed increase in murine cerebral blood flow[363] is contrary to that seen in many, though not all, human space and ground-based studies, as well as ground-based rat studies; indeed, the authors discuss the disparate findings seen across species and models.[363] Finally, although not isolated to the ventricles alone, atrophy of rat cardiac myofibers has been observed after 14 days of spaceflight.[399]

With respect to the rodent suspension model, structural and functional adaptations in the form of differential arterial remodeling have been seen in the HLU rat fore versus hind limbs, *e.g*. an increase in fore limb lumen diameter versus a decrease in hind limb sural artery.[61] In addition, with respect to the cerebrovasculature, two weeks of HLU induced an increase in rat basilar artery medial CSA and thickness, as seen in space-flown animals,[363] with a decrease in intraluminal CSA[62] and, interestingly, a potential impact from circadian rhythm dysregulation.[375] However, others have seen no change in maximal diameter, medial wall thickness or spontaneous tone of basilar arteries following a similar period of unloading,[363] contributing to misgivings regarding the validity of the suspended rodent as a model for microgravity-induced vascular deconditioning.[374, 443, 444] For example, fluid redistribution to the upper body has been demonstrated in rats within two hours of suspension,[445] and elevations in blood pressure and heart rate have been recorded.[374, 444] However, HLU of mice has induced a decrease in heart rate and only a slight (non-significant) increase in blood pressure.[373] Similarly, while some *ex vivo* studies of rat resistance-sized cerebral arteries demonstrated a reduction in the vasodilation response[407, 446] in parallel with an increase in

the myogenic tone, suggesting a potential mechanism, others have seen no effects on myogenic response.[363] Some mouse HLU studies have indicated alterations in the vasoconstrictor response, as well as altered baroreflex response,[373] whilst others, looking at *ex vivo* murine skeletal muscle arteries, have seen a diminution of vasodilation following HLU,[150, 402] with no effect on vasoconstriction.[150] Indeed, some have noted the broad range of, often contradictory, results seen in the literature, and have pointed to multiple factors that may contribute to inconsistencies, including experimental design, choice of species and strain, period of unloading and the choice of analytic techniques.[374] Importantly, differential findings with respect to a number of cerebrovascular endpoints have been seen when comparing murine HLU data to space-flown rats,[363] emphasizing the need for care when comparing data among models given the apparent species-specific differences in both structural and functional responses to microgravity, whether actual or simulated.

4.2.2. *Time Line of Progression*

Overall, defining the time line and extent of the various vascular events seen in astronauts under conditions of microgravity is hampered by conflicting data from small cohorts. Nonetheless, early physiological studies showed that a (one liter) reduction in leg volume is seen within 6—10 hours of reaching orbit,[447, 448] with a reduction in plasma volume taking place within 21 hours,[346] persisting as a 10—17% loss over both short- and long-term missions;[449, 450] this change in blood volume may affect astronaut oxygen uptake.[380] Cardiac distension occurs within the first two days in microgravity (possibly in parallel with an increase in intracranial pressure);[451] Blomqvist has suggested that there is an initial increase in LV size immediately on achieving orbit, but this is quickly followed (within 48 hours) by a persistent ~10% reduction in LV mass.[379, 452] Thickening of the intima-media wall (IMT) of both the carotid and femoral arteries can be seen within 15 days of attaining orbit[348] and remains elevated over long-term flights,[348, 428] although observation of this alteration has not always been statistically significant.[335] Post- versus preflight data also have demonstrated increased arterial stiffness after long-term flights.[324, 337]

Both cardiac output and stroke volume are increased during the first 24 hours in space and elevated for the first ~ten days.[453, 454] Indeed, emphasizing the rapidity of the acute cardiovascular response to fluid shifts from the lower body, a study of subjects undergoing parabolic flight demonstrated increases in cardiac output, heart rate and stroke volume within 20s of undergoing

zero gravity.[429] However, although some investigators have suggested that stroke volume[350] and cardiac output[349, 350, 428] remain progressively elevated over long-term flights, these findings are contradicted by results from Hamilton *et al*.,[455] who showed no change in either stroke volume or cardiac output over a 6-month flight, whereas Meck *et al*. and Herault *et al*. demonstrated decreases.[376, 456] Similarly dogged by conflicting results,[347] most studies describe a decreased heart rate during short-term flights,[457] with one study showing both heart rate and mean arterial pressure being reduced during the first 48 hours.[345] Other investigations have indicated that heart rate appears to normalize during longer flight times,[349, 350] although central arterial and systolic blood pressures may decrease at later time points.[343, 349, 350] It is important to draw attention to suggestions that the range in contradictory outcomes regarding even basic cardiac parameters may be a result not only of the choice of analytic techniques and small cohorts, but also the choice of preflight control values, in particular comparing inflight outcomes to preflight standing versus supine measurements[347, 350] and, possibly more importantly, the timing of the end-of-flight sampling relative to reentry.

Due to differences in their unloading dynamics, the most common ground-based human techniques, dry immersion and HDT bed rest, induce qualitatively differential effects on the vascular system in terms of response level and temporal kinetics,[384] with the immersion techniques generating responses that are more rapid and robust.[458] For example, one ground-based human study directly compared the acute cardiovascular responses following water immersion versus HDT bed rest,[383] and showed that water immersion induced a greater acute increase in left atrial diameter and stroke volume, although both mimicked that seen in space;[452] similar differential responses also have been seen with respect to increased cardiac output and decreased mean arterial pressure and heart rate.[383] However, all of these assessments were performed within 30 minutes of unloading, and no time points beyond 90 minutes of immobilization were utilized.[383] Of note, in at least one other head-to-head comparison, investigators compared short-term immersion (*e.g*. three days) versus medium-term (*e.g*. 21 days) HDT bed rest, suggesting interrogating variable durations may allow for better optimization and cross-model observations.[384]

Comparing physical vascular changes in astronauts versus those generated in ground-based models, investigators have seen a more gradual fall in plasma volume during HDT bed rest,[353, 393] albeit eventually reaching an equivalent ~15% decrease,[394, 459] and remaining low over a 100-day test period;[393] of note, Amirova *et al*. demonstrated a 14% decrease in plasma volume following

only three days of dry immersion.[384] Westby *et al*. described a 4% decrease in LV mass, first seen on day seven of HDT bed rest and continuing to a loss of ~15% over a 60-day study[394] and a similar loss of LV mass over equivalent study periods has been confirmed by others.[357, 379] However, some groups recorded no change in LV mass, even across 60—100 days of HDT bed rest.[393, 440] Unlike astronaut observations,[348, 428] Palombo *et al*. described seeing no change in femoral intima-media wall thickness (IMT) following 5-weeks of HDT bed rest, but instead saw a decrease in lumen diameter associated with inward remodeling;[387] interestingly, Navasiolova *et al*. saw an increase in femoral IMT after long-term (60 days) immobilization, but not short-term.[458] Both groups, as well as others,[387, 438, 458] have failed to see arterial stiffness, although vascular stiffness has been demonstrated in other HDT bed rest studies,[460, 461] including after only four days of dry immersion,[462] and changes in vasoconstriction and venous flow resistance have been seen as early as within seven[382] and four days of initiating HDT bed rest.[463]

Findings in ground-based human models with respect to stroke volume and cardiac output are consistent with cardiac deconditioning, although, interestingly, often contrary to the changes in parameters seen in astronauts: *e.g*. a decrease in stroke volume has been seen by days 4—7 of dry immersion[385, 462] or within the first month of HDT bed rest,[389, 390, 393] and maintained throughout long-term studies;[387, 438, 464] cardiac output is decreased by day 10—14 HDT bedrest,[389, 465] and maintained over 60 days HDT bed rest.[440] Three days of dry immersion also has been shown to be associated with decreased cardiac output, as well as decreased cerebral artery blood flow.[466] Similarly in contrast to most astronaut observations, heart rate appears to increase rapidly and significantly, observed by day one of HDT bed rest[391] and day seven following dry immersion,[385] then plateauing at an elevated level throughout both short-term[390] and long-term studies.[387, 438, 440] Nonetheless, a few investigators have demonstrated a decrease in heart rate, supporting heterogeneity in both space- and ground-based studies.[384, 467] For example, Amirova *et al*. demonstrated that three days of dry immersion induced a fall in systolic and diastolic blood pressures (BP), but no change in heart rate, whereas 30 days of HDT bed rest induced no change in blood pressure.[384] In another study, systolic BP was seen to fall after 12 days HDT bed rest and remained depressed for 21 days, although diastolic BP, mean BP and heart rate were unaffected;[435] in contrast, other have failed to demonstrate any change in BP parameters.[391, 392, 412] Thus, although the majority of review articles draw strong parallels between human space- and ground-based

cardiovascular models,[22, 27, 458, 468] one must appreciate the significant inter-individual heterogeneity seen under both conditions, including many of the basic vascular parameters.[164]

Of the publications scored for this report, only one provided information on the progression of vascular effects seen in animals during flight. Andreev-Andrievskiy *et al*. performed continuous monitoring of a small group (*n*=5/2) of flown mice, observing midflight changes in basic vascular parameters, *i.e*. blood pressure and heart rate.[366] During 30 days of flight, BP appeared unaffected, although heart rate rose after one week and remained elevated; however, we are unaware of any temporally equivalent astronaut data to allow for direct verification. In addition, there appeared to be little to no effect from the hypergravity of launch on murine BP, although heart rate decreased; interestingly, descent induced dramatic falls in both parameters, with values normalizing within >one hour of landing.[366] In addition, a decline in plasma volume has been observed at landing in rats following an eight day flight.[469] An early study of telemetric readings from rats during 20 secs of parabolic flight revealed rapid cardiovascular changes, with an increase in heart rate during hypergravity, but a reduction during microgravity, a 7% increase in mean arterial pressure, and a non-significant (–13%) decrease in central venous pressure.[470] However, the relevance of these findings in light of the overall paucity of data and the brevity of the induced microgravitational conditions compared to spaceflight is unclear.

Greater interrogation of vascular progression has been performed in the animal ground-based versus spaceflight studies. For example, looking at changes in organ weight, investigators have intimated that rats experience fluid redistribution to the upper body within two hours of suspension.[445] In addition, Colleran *et al*. demonstrated a reduction in femoral and tibial perfusion within ten minutes of unloading, with femoral blood flow remaining depressed through 28 days HLU.[371] In contrast, blood flow to the upper skeleton (skull, mandible, scapula) was increased acutely following ten minutes of unloading, but subsequently returned to, and remained at, baseline between 7 and 28 days HLU.[371] However, heterogeneity in the effects seen in basic human vascular parameters is again prevalent in animal studies. For example, one review suggests that increases in blood pressure and heart rate, measured using direct aorta cannulations, have been seen as early as 1—3 days following unloading,[444] with Zhang *et al*. demonstrating increased heart rate, systolic and diastolic BP by the end of a 28-study of rat HLU;[374] however, others saw no changes in either BP or heart rate in studies conducted over 1—28 days.[372, 406, 471, 472] Furthermore, Powers *et al*. demonstrated a moderate decrease (-11%) in murine heart rate over the first two days of

suspension, but with normalization by day six, and a slight, non-statistical increase in mean arterial BP.[373] Of interest, the group discussed differences with respect to the vascular response to simulated or actual microgravity, seen not only between human and rodents, but also between rats and mice, suggesting that some of the observed differences may reflect not only the relative circulatory adjustments made by bipeds versus quadrupeds, but also the significantly smaller size (volume) of rodents exerting a more limited effect on fluid distribution.[373]

4.2.3. *Recovery Kinetics*

Although multiple cardiovascular assessments have been conducted on astronauts following their return, establishing clear time lines of recovery is hampered by disparate collection points and few sequential analyses. Early investigators showed that the majority of the shifts in fluid distribution that occur rapidly in astronauts during flight appear to begin resolution equally rapidly on return.[473] For example, 65% of the postflight compensatory increase in leg volume is completed within 90-150 minutes of landing,[425, 447] and completes resolution over the next 2—6 days.[450, 473] Fluid metabolism also appears to normalize within one week after short flights,[346] although longer spaceflights require more prolonged recovery periods.[451] However, as with the previously described inflight effects, the vascular postflight read-outs vary significantly, likely due to both individual variability and the variable lag times between landing and sampling combined with the rapidity of normalization. Of the other basic parameters, an increase in heart rate is consistently seen postflight; this has been demonstrated anywhere from four to 48 hours after landing following both short-term[345, 378, 419] and long-term[337, 343] flights, with at least one study indicating a slight elevation persisting through recovery days 7—14.[378] Whilst two studies, with measurements taken soon (within hours) after landing following short-term flights, indicate a decrease in stroke volume,[378, 419] other assessments performed >24 hours post-landing after long-term flights have shown a slight increase or no change in stroke volume relative to preflight.[337, 349] Thus, given the discrepancies in flight times and sampling points, it is unclear whether the apparent rapid return to preflight levels occurred during the longer flights, *i.e*. as an adaptation, or normalized during the course of the first 24 hours post-landing. A similar lack of clarity can be ascribed to increases seen in systolic and diastolic BPs, as well as mean arterial pressure, observed relative to both pre- and inflight values at 4 hours—2 days after short-term flights,[345, 378, 409, 419] since others have seen no change relative to preflight values when sampled at 24 hours after long-term flights.[337]

Importantly, any increases in mean arterial pressure and mean systemic vascular resistance appear to return to baseline 1—2 weeks postflight,[378] with at least one study suggesting that normalization may occur within 4—6 hours of landing.[343]

With respect to the physical changes, the reduction in LV mass observed within 2—12 hours after landing[377, 379] has been shown to completely recover by recovery day three, although one study has suggested that left ventricular function, measured by end-diastolic volume index and stroke volume index, remained below baseline until at least 1—2 weeks later.[378] There are indications that carotid artery stiffness[337] and carotid IMT[348] remains increased for days following flight, although markers of oxidative stress and inflammation associated with vascular resistance have returned to preflight levels within the first week of recovery.[335] Overall, although there are hints that the duration of flight may affect the speed of recovery,[474] in general, our understanding of the persistence of vascular effects postflight and the kinetics of recovery following a return to one G are unclear in the astronaut cohort.

The kinetics of vascular recovery following immobilization in human ground-based studies have formed the basis for countermeasure development for astronauts despite the limitations on model correlation and lack of temporally sequential assessments. For example, extrapolation from ground-based bed rest studies led to the use of saline loading as a countermeasure to orthostatic intolerance.[475] However, although fluid shifts may be critical to many of the altered hemodynamic parameters of interest, some of the critical basic values have not been widely assessed in ground studies, especially over the long-term. For example, despite an extensive literature search, few ground-based studies have addressed the kinetics of plasma volume restoration,[476] although some have suggested that plasma volume recovers by day three of reambulation following 60-days of HDT bed rest[394] or day four following 7-days of dry immersion.[477] As observed in astronauts, an increased heart rate has been seen following reambulation after long-term (60 days) HDT bed rest; however, unlike in astronauts, such studies have indicated a failure to complete normalization by reambulation days three,[412] four,[440] eight,[391] ten,[390] 12,[478] and 15,[412] respectively, or by reambulation day five following 21-days of dry immersion.[385] Interestingly, following varying terms of bed rest (17 and 42 days), heart rate returned to baseline by reambulation day four[479] and 32,[480] respectively, again suggesting the potential for a duration-dependent recovery period. Addressing changes in blood pressure, following 60-days HDT bed rest, one study showed that all parameters had returned to baseline by reambulation day four,[440] although other studies have

indicated a delay in the recovery of mean arterial pressure until day 12.[478] With respect to physical changes, 60-days of HDT bed rest induced a reduction in LV volume and mass;[394] after three days of reambulation, LV volume had returned to baseline, although mass remained reduced.[394]

Limited data are available on the vascular recovery kinetics of either space-flown or ground-based unloaded animals. One study showed that, after 30 days spaceflight, heart rate in a small group (n=2) of mice was elevated and had not fully returned to baseline by recovery day seven, although blood pressure did not differ from controls during the same period.[366] In contrast, other investigators have suggested that recovery of both parameters occurs within hours of re-ambulation, albeit with regional blood flow instability.[444, 481] As seen in human studies, Powers *et al*. demonstrated that, after 14 days of HLU, mice showed an immediate increase (15%) in heart rate above baseline following reambulation, however no subsequent time points were assessed.[373] Also similar to observations in human studies, other groups have demonstrated a reduction in the mesenteric vasoconstrictive response,[471, 482, 483] seen both in rats and mice and persisting for ~one day following 18-[361] or 15-day[360] flights, respectively, as well as in gastrocnemius[398] and cerebrovascular[363, 401] arteries, with one of the groups demonstrating a return to baseline levels by recovery day five.[360] These data suggest a mechanism for the observed decrease in peripheral vascular resistance,[398] also offering an explanation for the orthostatic intolerance seen postflight.

4.2.4. *Potential Mechanisms*

Early hypotheses put forward to explain the vascular effects seen in astronauts, in particular the orthostatic intolerance, focused on the rapid and dramatic reduction in plasma volume, with some suggesting that extravasation occurred into upper body interstitial spaces due to the headward fluid shift.[451, 484] Others have noted the progressive decrease in extracellular fluid volume seen during the first week of microgravity, together with a lack of change in total body water content, indicating an increase in intracellular fluid volume, possibly due to increased permeability of capillary membranes.[346, 362] In addition, data from both human space and bed rest studies,[344, 485] as well as from space-flown animals,[381] have suggested that changes in plasma volume may be associated with alterations in cerebral autoregulation, with the change in plasma volume likely being causative rather than secondary. Interestingly, various cerebral arteries and veins display heterogeneity in their responses to simulated microgravity,[434, 436] a possible reflection of differing regulatory roles played by various areas of the brain.[332] Indeed, the role played by neural

controlling mechanisms in vascular responses, such as the baroreceptor function, has been a focus of multiple investigation and discussion for decades, in both human and animal models.[372, 382, 395, 486, 487] Nonetheless, currently, the mechanism(s) underlying the fluid redistribution phenomena remains undetermined,[488] especially given the lack of clarity over the presence[489] or not[346] of diuresis.

In general, ethical considerations have forced most of the vascular-related mechanistic studies to be performed in animal models instead of humans. However, the variation in results due the range of animal species and strains, analytic techniques and endpoints have impacted our ability to draw clear conclusions. For example, work with HLU rats has demonstrated differential arterial remodeling in the fore versus hind limbs following two and four weeks of unloading, posited by the authors as the result of reductions in transmural pressure and wall shear stress.[483] However, others have suggested that changes in wall shear stress cause atrophy of arterial smooth muscle cells,[62] leaving us with a "chicken-or-the-egg" conundrum. Several investigators looking at rat resistance arteries in HLU animals have suggested that the observation of an increase in myogenic tone, causing a reduction in the vasodilation response, was a potential defense mechanism against rises in perfusion pressure, a process likely mediated through endothelial-dependent NOS signaling;[63, 150, 404, 405, 407, 446, 490] the vasoconstrictor hyporesponsiveness seen in many rat arteries also has been attributed to hemodynamics.[403] Indeed, low peripheral vascular resistance, in combination with dysfunction of the baroreceptor reflex complex, has been proposed by some to be one of the chief pathophysiological mechanisms underlying orthostatic intolerance.[16, 491]

Importantly, investigations of cardiac and vascular tissues from space- and ground-based animal studies have indicated altered regulation of genes associated with oxidative stress,[400, 405] cell cycle,[400, 492] senescence,[400] cell death, immune response, and metabolic stress,[362] some of which also have been observed in astronauts,[428] with evidence of persistent marker expression during the recovery period.[368] It is anticipated that continuing such studies eventually may shed light on the more fundamental mechanisms underlying vascular effects; for example, cross-validation through analysis of astronaut and animal samples has indicated a spaceflight-associated miRNA signature, shared by rodents and humans, and found to regulate vascular damage.[493] Such links are essential in order to validate models and assist in the design of more scientifically rational investigations. Another important observation followed a data comparison of animals sacrificed in

space[365] versus a similar cohort sacrificed within three hours of landing.[397] The investigators identified cytoskeletal changes unique to the "readapted" group and suggested that the early period of readaptation may, in fact, be more damaging to the heart than the spaceflight itself,[365] emphasizing the need for inflight versus postflight sampling, depending on the endpoint of interest.

5. Central Nervous System (CNS) Outcomes

Astronauts have described issues with learning and memory,[494, 495] as well as neurovestibular disturbances during flight and on return,[14, 496] raising concerns regarding the impact of spaceflight on the brain. Subsequently, it has been shown that the astronaut brain does, indeed, undergo changes in structure and function as a consequence of fluid shifts and changes in intracranial pressure (ICP),[497] with acute and progressive adaptive compensations to weightlessness experienced during flight.[498] Since there is the potential for alterations in the cerebellum, cortical sensorimotor, somatosensory areas and vestibular pathways[6] that might manifest in the form of behavioral changes at the sensory, motor and cognitive levels, both during and postflight, including significant ocular abnormalities,[499] there is a need to address significant gaps in our understanding of microgravity-induced changes under mission conditions, including the effect of flight duration, *etc*.[9] Of note, as with the other systems considered in this report, difficulties in discriminating the specific role of microgravity on cerebral dysfunction from other stressors has proven difficult.[500]

5.1. Publications Overview

5.1.1. *Astronaut Category* (**Table 9**)

- A large scientific literature indicates that there is a sex-specific risk for the development of brain disorders, with a growing consensus that the estrogen hormone family provides neuroprotection.[501] Nonetheless, with respect to astronaut studies, 11/25 of the scored publications did not describe the gender ratio in their cohort characteristics; of the remainder, the majority of subjects (160/192) were male and no study looked at sex as a factor.
- Despite NASA concerns over long-term CNS effects from exposure to the space environment,[6] albeit primarily with respect to radiation exposure rather than microgravity,[502-504] the majority of the scored publications involved only acute postflight analyses of the astronaut cohort, with only four taking inflight samples.[505-508] This deficit possibly speaks to an operational focus on immediate mission impact rather than delayed (postflight) outcomes, although it also may reflect an overall unwillingness on the part of astronauts to undergo testing that has the potential to compromise their future flight availability. In addition, only 4/25 publications carried out longitudinal analyses[505, 507-510] and another surveyed postflight subjects at non-specified recovery time points.[511]

5.1.2. *Ground-Based Human Category* (**Table 10**)

- As described in **section 4.1.2**., the toxic effects of raised carbon dioxide levels (hypercapnia) have been known for almost a century.[512] Due to practical constraints, CO_2 levels within spacecraft range over 2.3—5.3 mm Hg[513] and, therefore the effect of hypercapnia on astronauts has been investigated. Initially, research focused on its potential role in the headaches experienced on the ISS,[514] although it is now being interrogated for other possible effects, induced either independently or in combination with other stressors, on cardiorespiratory,[515] ocular,[356, 516] and behavioral changes.[517] Indeed, 11 of the 28 publications scored in the CNS-human immobilization category included hypercapnia as a condition in their experimental design.[354, 396, 517-524]
- Although ten publications included mixed sex cohorts, only one addressed gender as a factor,[525] one involved an older, more age-appropriate cohort,[520] and ten described the fitness/training status of the subjects. Ten publications included a countermeasure arm, usually in the form of exercise.
- Although nearly all of the studies performed intermediate measurements during the course of bed-rest, none looked at effects later than two weeks post-reambulation.

5.1.3. *Animals in Space Category* (**Table 11**)

- All studies considered in this category used rodent models, with a ratio of 4:8 rat to mouse subjects; all of the murine studies used C57BL/6 and all of the rat studies used Sprague-Dawley.
- Only one of the studies had a mixed sex grouping;[526] it addressed the effects of the space environment on brain development so that the studies involved pups (with their dams), but did not consider sex as a factor in the outcome. Otherwise, the remaining scored studies used single gender (7:4, male:female). Only one study employed age-appropriate animals,[527] with the remaining ten publications using juveniles.
- All studies employed a matched ground-control group, with some including an additional matched vivarium cohort.
- Most of the studies focused on acute (within 48 hours) recovery time points, with only two conducting inflight analyses[526, 527] and one looked at delayed recovery (day 27).[526]

5.1.4. *Ground-Based Animal Category* (**Table 12**)

- All of the 19 studies considered in this category used rodent models, with a ratio of 11:8, rat to mouse subjects; all of the murine studies used C57BL/6.
- The emphasis placed by NASA on the investigation of radiation-induced late brain-related deficits through ground-based studies likely encouraged the combined radiation-microgravity experimental conditions seen in 6/19 publications,[59, 362, 528-531] with several including late post-reambulation time points.
- Four of the 19 scored studies considered in this category used female subjects,[59, 530-532] with none considering sex as a factor. Five of the scored studies had used age-appropriate animals, with all but one[532] of the remainder using juveniles.
- Only two studies looked at time points during unloading, neither of which involved the combined conditions.[533, 534]

Table 9: Highest scoring publications in the astronaut CNS category.

ASTRONAUT: CNS	Environment parameters	Astronaut characteristics	Science / time points	Score	Main Scientific Findings
BRAIN STRUCTURE					
Short (≤30 days) flight					
Lee JK *et al.* 2019[535]	9	4	5	18	Short (≤30 days) vs. long (~6 mo) flight: Pre- vs. postflight white matter changes using dMRI†. Upward shift of brain
Roberts DR *et al*. 2017[433]	9	3	4	16	Short (~14 days) vs. long (~6 mo) flight: MRI showed narrowing of central sulcus, upward shift of brain, narrowing of CSF†† spaces
Riascos RF *et al*. 2019[536]	9	3	4	16	Short (~30 days) vs. long (~6 mo) flight: Pre- vs. postflight grey and white matter changes using qMRI††† associated with neuroplasticity
Koppelmans V *et al*. 2016[432]	9	2	4	15	Short (~14 days) vs. long (~6 mo) flight: Retrospective MRI analysis: decrease in gray matter volume in temporal and frontal lobes; increase in medial primary somatosensory and motor cortex associated with neuroplasticity
Long flight (≥4 months)					
Kramer LA *et al*. 2020[509]	9	3	6	18	~170 days flight: Longitudinal MRI study (1-360 days): increased brain and CSF volumes persisted up to 1 yr
Alperin N *et al*. 2017[537]	9	2	5	16	Short (~14 days) vs. long (~5 mo) flight: White matter hyperintensity showed increase in periventricular areas
Marshall-Goebel K *et al*. 2021[538]	9	3	4	16	Mean 191 days flight: MRI suggested positive correlation among lateral ventricle volume, optic disk edema and retinal thickness
Roberts DR *et al*. 2019[539]	9	3	3	15	Short (~15 days) vs. long (~162) flight: Long, but not short, flight induced increase in ventricular volume. 3 white matter regions associated with cognitive changes (bilateral optic radiations; splenium of corpus callosum)

NEUROVESTIBULAR/NEUROOCULAR (SANS)					
Short flight (≤30 days)					
Kramer LA *et al*. 2012[511]	9	2	2	13	<30 versus >30 days flight: qMRI of postflight astronauts showed spectrum intra-orbital and intracranial changes
Long flight (≥4 months)					
Marshall-Goebel K *et al*. 2021[538]	9	3	4	16	Mean 191 days flight: MRI suggested positive correlation among lateral ventricle volume, optic disk edema and retinal thickness
Mader TH *et al.* 2011[540]	9	3	3	15	~6 months flight: OCT*and MRI showed spectrum of optical changes (survey: 29% and 60% of short vs. long duration astronauts experience visual degradation)
Rohr JJ *et al*. 2020[510]	8	0	6	14	~6 months flight: qMRI did not show distension of optic nerve postflight in limited group; questioned association with increased ICP**
Patel N *et al*. 2018[541]	9	2	3	14	~6 months flight: OCT shows disc edema-like changes in optic nerve and tissue; role of ICP questioned
SENSORINEURAL/COGNITIVE					
Short flight (≤30 days)					
Lowrey CR *et al*. 2014[542]	9	2	4	15	12—16 days flight: Assessment of impact of foot sole sensitivity on balance during recovery. Differential big toe vs. heel loss vs. hypersensitivity suggested targeted reweighting on day 3 postflight
Long flight (≥4 months)					
Tays GD *et al*. 2021[507]	9	3	8	20	~188 days flight: Cognitive and sensorimotor testing pre-, inter- and postflight. Greatest effect on sensorimotor; recovered by day 30 postflight
Harris LR *et al*. 2017[505]	9	3	7	19	~168 days flight: Perceptual upright depends on visual, gravity and idiotropic cues. Astronauts showed shift in cue-weighting during flight that persisted chronically on return
Cebolla AM *et al*. 2016[506]	9	4	5	18	6 months flight: EEG*** recordings pre-, during and postflight suggested microgravity leads to heightened visuo-attentional state (motor cortex) with increased

					demand for integration from cerebellum and vestibular networks
Mulavara AP *et al*. 2010[543]	9	3	6	18	~185 days flight: Assessed locomotor dysfunction and recovery using functional mobility test (FMT). All showed locomotor dysfunction with 95% recovery at R15
Takács E *et al*. 2021[508]	9	3	6	18	~6 months flight: Assessing visuospatial performance, showed astronauts to be slower and more error-prone, with diminished attentional resources
Mulavara AP *et al*. 2018[256]	9	4	4	17	~159 days flight (compared to 70 days bed-rest+/- exercise): Deficits in static and postural stability after both spaceflight and bed rest, with no mitigation with exercise
Cohen HS *et al*. 2012[544]	9	3	3	15	~6 months flight: Compared Sensory Organization test battery versus FMT to assess astronaut standing balance. Determined both are needed to assess astronaut balance deficits

† dMRI: Diffusion magnetic resonance imaging (MRI)
†† CSF: cerebral spinal fluid
††† qMRI: Quantitative/qualitative MRI
* OCT: optical coherence tomography
** ICP: intracranial pressure
*** EEG: electroencephalograph

Table 10: Highest scoring publications in the ground-based human-CNS category.

HUMAN HDT STUDIES: CNS	Environment parameters	Astronaut characteristics	Science / time points	Score	Main Scientific Findings
BRAIN STRUCTURE					
Short-term (≤10 days HDT)					
Kramer LA *et al*. 2017[354]	4	3	4	11	7 days HDT (12°)±CO_2: SPACECOT study: MRI indicated alterations in cranial anatomy and physiology, enhanced by brief exposure to CO_2
Marshall-Goebel K *et al*. 2017[545]	4	2	3	9	5 hrs HDT (6°-18°)±CO_2±LBNP†: MRI of different HDT conditions: increase in orbital and extracranial CSF††
Medium-term (10—30 days HDT)					
Lee JK *et al*. 2021[396]	3	2	8	13	30 days HDT±CO_2: Global upward shift of the brain with free water redistribution, exacerbated by CO_2.
NEUROVESTIBULAR/NEUROOCULAR (SANS)					
Short-term (≤10 days HDT)					
van Oosterhout WPJ *et al*. 2015[546]	3	3	7	13	3 x 5-days HDT: induction of "space headaches", primarily on HDT day 1. Countermeasures provided some mitigation
Prakash M *et al*. 2015[547]	3	2	5	10	3 x 5 days: HDT did not precipitate SMS†††, although small/large intestinal transit was accelerated
Kermorgant M *et al*. 2021[548]	2	2	6	10	5 days dry immersion: Increased optic nerve sheath diameter and greater thickness of the retinal nerve fiber layer
Laurie SS *et al*. 2017[521]	3	2	4	9	2 x 1 hr ±CO_2: HDT increased both IOP and ICP*. CO_2 did not augment changes
Medium-term (10—30 days HDT)					
Taibbi G *et al*. 2016[549]	3	3	5	11	14 vs. 70 days HDT: Time-dependent peripapillary retinal thickening. Slight IOP increase recovered post-HDT bed rest
Long-term (≥50 days bed rest)					

Taibbi G *et al*. 2017[550]	3	3	4	10	70 days HDT±exercise: Peripapillary retinal and circumpapillary nerve fiber layer thickening, but no optical disk edema. No mitigation with exercise
SENSORINEURAL/COGNITIVE					
Short-term (≤10 days HDT)					
Basner M *et al*. 2018[520]	3	3	4	10	26.5 hrs HDT (-12º) ± CO_2: HDT induced faster cognitive responses, but less accuracy. CO_2 reversed response changes
Clement G *et al*. 2015[551]	2	2	5	9	5 days HDT±centrifugation: Subjective decrease in neuro-vestibular symptoms with centrifugation
Medium-term (10—30 days HDT)					
Salazar AP *et al*. 2021[518]	4	2	8	14	30 days HDT±CO_2: Using fMRI, showed differential periods of adaptation. No CO_2 effect
McGregor H et al. 2021	3	3	7	13	30 days HDT±CO_2:
Banker LA *et al*. 2021[519]	4	2	6	12	30 days HDT+CO_2: Using MRI/sensorimotor adaptation test. Showed greater reliance on procedural (implicit) memory processes. 5 presented with optic disk edema (symptom of SANS***)
Macaulay TR *et al*. 2016[525]	3	4	4	11	30 days HDT±exercise with LBNP; men vs. women: LBNP attenuated loss of balance control in men, but not women
Long-term (≥50 days HDT)					
Yuan P *et al*. 2018[552]	2	3	8	13	70 days HDT: Activation in the bilateral insular cortex occurred across study.
Salazar AP *et al*. 2021[518]	3	2	6	11	60 days HDT: fMRI showed differential changes dependent on period of adaptation (acute v late)

† LBNP: lower body negative pressure
†† CSF: cerebrospinal fluid
††† SMS: space motion sickness
* IOP and ICP: intraoptical and intracranial pressure
** fMRI: functional MRI
*** SANS: space-associated neuro-ocular syndrome

Table 11: Highest scored publications in the space animal-CNS category.

ANIMAL-SPACE STUDIES: CNS	Environment parameters	Astronaut characteristics	Science / time points	Score	Main Scientific Findings
BRAIN STRUCTURE					
Short flight (~30 days)					
Davet J *et al*. 1998[553]	9	0	5	14	14 days flight (flight vs. HLU): Compared recovery times (6 hrs vs. 2 days) for choroid plexus changes. Persisted >2 days
Gabrion J et al. 1996[554]	9	0	4	13	13 days flight: inflight analysis of choroidal organization showed alterations in the fine structure of choroid plexus, consistent with a marked reduction in CSF production
Blanc S *et al*. 1998[555]	8	1	3	12	17 days flight: Space flight exerted inhibitory effect on serotonin metabolism Probably through HPA† axis). Unable to distinguish between stressors, microgravity vs. landing
Mao XW *et al*. 2020[333]	7	0	4	11	35 days flight: Increase in hippocampus apoptosis. Disruption in BBB†† integrity; potential for long-term neurovascular damage
Latchney S et al. 2014[556]	7	0	4	11	13 days flight: Increase in olfactory bulb volume and neuroblasts, decrease in apoptotic cells seen only in ground-based AEM cohort
NEUROVESTIBULAR/NEUROOCULAR (SANS)					
Short flight (~30 days)					
Mao XW *et al*. 2019[362]	8	0	6	14	35 days flight: IOP††† lower post- v. preflight. Increase in retina and retinal endothelial cell apoptosis. Disruption of blood-retinal barrier
Mao XW *et al*. 2018[557]	9	0	4	13	35 days flight: Increase in retina and retinal endothelial cell apoptosis. Proteomic analyses showed upregulation in multiple pathways
Overbey EG *et al*. 2018[558]	7	0	3	10	35 days flight: Decline in retinal performance recorded by thinning of retina, retinal pigment epithelium and choroid layer
Mao XW *et al*. 2013[559]	7	0	3	10	13 days flight: Evidence of increased mitochondrial apoptosis in retina

SENSORINEURAL/COGNITIVE					
Short flight (~30 days)					
Ronca AE *et al*. 2019[527]	7	3	5	15	20—35 days flight: Video observation of mouse behavior showed rapid adaptation; potential development of stereotypic movements
Kwok AT *et al*. 2020[560]	7	0	6	13	35 days flight: Comparing gait characteristics among flight v. ground-control v. vivarium: significant changes in majority of hind- and forelimb gait characteristics in flight mice
Temple MD *et al*. 2002[526]	6	0	5	12	16 days flight: Cognitive testing of P8 v. P14 litters v. ground controls. Little to no differences between flight and ground groups
Yamasaki M *et al*. 2004[367]	6	1	4	11	16 days flight: Development of aortic nerve and baroreflex system in 9-day old neonates. 50% mortality inflight. Decrease in numbers of unmyelinated aortic fibers; similar to HLU

† HPA: hypothalamic-pituitary-adrenal axis
†† BBB: blood-brain barrier
††† IOP: intraocular pressure

Table 12: Highest scoring publications in the ground-based animal-CNS category.

GROUND-BASED ANIMAL STUDIES: CNS	Environment parameters	Astronaut characteristics	Science / time points	Score	Main Scientific Findings
BRAIN STRUCTURE					
Medium-term (10—30 days)					
Mao XW *et al*. 2016[59]	5	3	4	12	21 days HLU +/- LD/DR† gamma: Acute (7 days post-) increase in OS†† markers (lipid peroxidation) and late reduction (9 months) in microvessel density
Mao XW *et al*. 2017[530]	5	3	3	11	21 days HLU +/- LD/DR gamma: 1 month recovery: increase in OS markers in HLU and hippocampus apoptosis; differential increase in combined stress group
Overbey EG *et al*. 2019[531]	5	3	3	11	21 days HLU +/- LD/DR gamma: 4-months recovery: DEG††† analysis showed greatest effect in combined group in pathways associated with neurogenesis, neuro-plasticity, neuropeptide regulation
Frigeri A *et al*. 2008[561]	4	3	2	9	2 weeks HLU: Significant changes in gene regulation following HLU; pathway analysis showed impact on synaptic plasticity and learning processes
NEUROVESTIBULAR/NEUROOCULAR (SANS)					
Medium-term (10—30 days)					
Mao XW *et al*. 2019[562]	5	4	4	13	14 days HLU +/- LD/DR gamma: Increase in retinal apoptotic cells and OS markers in both radiation groups; highest in combined
SENSORINEURAL/COGNITIVE					
Medium-term (10—30 days)					
Raber J *et al*. 2021[528]	6	4	5	15	30 days HLU +/- GCRsim* radiation: HLU had differential effects when combined with radiation; effects seen behaviorally and in metabolic pathways
Bellone JA *et al*. 2016[529]	5	4	6	15	3 weeks HLU +/- LD/DR gamma: HLU affected exploratory/risk-taking behaviors; combined stressors affected BBB** integrity

† LD/DR: low dose/dose rate

†† OS: oxidative stress

††† DEG: differential gene expression
* GCRsim: 5-ion simulated beam of galactic cosmic radiation (see [563, 564])
** BBB: blood-brain barrier

5.2. CNS Microgravity Studies: Data Comparisons Across Models

A number of neurological, ocular and behavioral symptoms have been experienced and described by astronauts, such as impaired cognitive function, in- and postflight headache, and visual impairment, both during and after flight. The potential impact of these issues on mission execution has prompted significant concern and a need to understand their etiology.

5.2.1. *CNS-Associated Outcomes*

5.2.1.1. *Structural changes*: The significant gaps in our understanding of space-induced CNS effects, as with the other fields of interest described in this report, reflect the small group sizes in astronaut studies and paucity of inflight data, particularly following long-term missions.[494] Nonetheless, a number of astronaut and cosmonaut studies, comparing pre- to postflight data, have indicated that the brain undergoes physical and structural changes during spaceflight.[497] For example, using functional magnetic resonance imaging (MRI), increased volumes have been seen in the mean summated brain (gray plus white matter),[509] lateral ventricles,[536, 538] and the cerebrospinal fluid (CSF) in the optic nerve sheaths postflight,[509, 565] albeit not consistently,[537] and with indications that such effects persist and progress with mission duration.[566] Observation of other mission duration-dependent physical changes have included: increased incidence rate of narrowing of the central sulcus;[433] an increase in free water redistribution[535] reflecting upward shifting of the brain;[433] an increase in periventricular white matter hyperintensity,[537] which has been associated with inflammation and vascular risk;[567] regionally differential white matter alterations, possibly reflecting vestibular and proprioceptive processing changes;[535] a narrowing of CSF spaces[433] and parenchymal crowding[539] at the vertex; an increase in pituitary deformation;[509] and an increase in total ventricular volume (which also has been negatively associated with age).[539] In addition, using T1- or T2-weighted MRI, extensive gray matter volumetric decreases have been seen in large areas covering the temporal and frontal lobes and around the orbits,[431, 432] with additional bilateral focal gray matter increases in the medial primary somatosensory and motor cortex;[432] these regions represent lower limb control and, therefore, these changes potentially reflect neuroplasticity. Overall, a host of gray and white matter alterations have been observed following spaceflight,[535, 536] although their specific role in the development of symptomatic CNS outcomes is not clear. As will be discussed below, the majority of hypotheses put forward with respect to the induction of the physical CNS alterations highlight

adaptive responses to microgravity-induced cephalad fluid redistribution (**see 5.2.4. *Potential Mechanisms***).

Many of the ethical restrictions that limit brain structural analysis of astronauts naturally also apply to ground-based human studies. Nonetheless, following 30-day HDT bed rest, Lee *et al*. showed that subjects exhibited a similar global upward shift of the brain with associated free water redistribution,[396] as described above. An increase in lateral ventricle volume also has been seen following HDT bed rest, which appears exacerbated by concomitant exposure to increased ambient (0.5%) CO_2,[354] a condition frequently experienced on ISS.[514] Interestingly, exposure to increased CO_2 exacerbated some, though not all, of the observed regional gray matter volume changes.[535] A significant decrease in functional connectivity among vestibular, motor and primary visual brain areas following bed rest has been confirmed by several groups,[517, 568] with McGregor *et al*. also demonstrating differential connectivity changes in the presence of CO_2;[517] exercise appeared to mitigate the functional connectivity decrease.[569]

Although some of the structural changes seen in ground-based studies are qualitatively similar to those seen following spaceflight, significant differences also are evident. For example, following ~60 days of HDT bed rest, Roberts *et al*. showed only an upward shift of the central mass of the brain, failing to see any changes in gray matter, white matter, CSF or ventricle volumes.[570] Similarly, despite demonstrating regional-specific free water changes following 60 days of HDT bed rest, Koppelmans *et al*. saw no white matter microstructural alterations.[571] Furthermore, although some groups have seen differential changes in gray matter volume following HDT bed rest, with both regional-specific increases and decreases,[396, 572, 573] this has not been seen by all.[570] Others have noted differential changes in compartmental free water distribution in white matter, with increases in fractional anisotropy and axial diffusivity, and decreases/no change in radial diffusivity,[522, 535] with exaggerated responses when simultaneously exposed to CO_2.[535] Significantly, these observations are reversed in astronauts,[535, 536] possibly reflecting a form of adaptation specific one or both of the conditions.[572] Overall, it appears that many of the CNS microstructural changes described during and following spaceflight have not been reported in human ground-based studies. Whether this discrepancy is due to the lack of sufficient comparative astronaut data, incomplete removal of gravitational effects, or a failure to include other spaceflight stressors (*e.g*. space radiation, sleep disturbance, *etc*.) is unknown.

Few spaceflight studies conducted on rodents have assessed gross structural changes in the brain; where anatomical or pathological analyses have been conducted, the focus has tended to be at the cellular or subcellular levels. For example, one study of 17-day flown rats showed significant depletions of 5-hydroxytryptamine in selective nerve terminal regions of the brain, an indication that a complex form of stress may be affecting serotonergic neurons.[555] A proteomic analysis of brain tissue of 13-day flown mice indicated significant alterations in proteins of both white and gray matter related to neuronal structure and metabolic function.[574] However, one interesting investigation did look at gross structure, examining relative volume changes in the olfactory bulb (OB) and comparing space-flown to two ground-control cohorts of mice.[556] One of the ground-based cohorts was housed in AEMs (**see 1.1.3. *Animal studies in space***) and demonstrated increased OB volume, a higher number of OB neuroblasts and fewer apoptotic cells than vivarium controls; however, these changes were not seen in the space-flown cohort, who instead exhibited a greater density of apoptotic cells. Inflight (13 days) sacrifice of rats enabled examination of the choroid plexus, a network of blood vessels in the ventricles that are chiefly responsible for the secretion of CSF. Ultrastructural analysis showed significant disorganization, consistent with a decrease in CSF.[554]

There is a similar paucity of gross structural studies using ground-based models. Salehi *et al*. examined brain volumes associated with motion (motor cortex) and spatial learning and memory (hippocampus) in rats and failed to see any changes following 14 days of HLU.[575] Similarly, Chen *et al*. observed no apparent structural change in the hippocampus, cerebral cortex or striatum of rats after seven days of HLU, although, following 21 days of HLU, atrophy of regional neurons in the cerebral cortex was seen, suggesting a potential time-dependent effect.[576] As described with the space-flown animals, the majority of HLU pathological analyses have concentrated analysis at the cellular or subcellular levels, with protein and gene analysis of brain tissues, including the hypothalamus[577, 578] and hippocampus,[492, 530, 532, 578] indicating only non-specific changes in synaptic plasticity and learning processes,[561, 578] oxygen homeostasis,[492] neuroinflammation,[532] *etc*.

5.2.1.2. *Neurovestibular/Neuroocular (Space-Associated Neuroocular Syndrome [SANS])*: The previously-described harbinger of space-induced CNS injury, the headache, has frequently been included as part of space motion sickness (SMS), a term used to describe an array of

neurovestibular symptoms commonly experienced within the first 72 hours of entry into, and return from, space.[579] SMS involves loss of appetite, nausea, vomiting, headache, impaired concentration, cold sweating, fatigue, increased breathing rate, impaired mental and physical performance, disorientation, and lethargy,[580] and has affected ~70% of astronauts.[579] However, it is not known whether headaches experienced later during missions, *i.e*. outside the 72 hour window, are a separate entity, as proposed by some,[328] and, if so, potentially an outcome from increased ICP;[581] this conclusion is extrapolated from the similar effects seen with intracranial hypertension.[511, 582, 583] Despite the observation that, as the length and number of flights has grown, there has come a prevalence for overlapping headaches with visual disturbances,[582] a relatively recent review clearly distinguishes between intracranial hypertension and the ocular changes seen in spaceflight.[584] However, although both headaches and visual disturbances have been independently associated with chronically elevated ICP,[585, 586] we were unable to identify observation of inflight astronaut ICP, likely due to the invasive nature of ICP monitoring,[581] with a small study looking at parabolic flight subjects showing no pathological elevation of ICP.[587] Significantly, headache is frequently experienced during the first day of HDT bed rest,[546] and other SMS-like neurovestibular symptoms also being experienced within the first few days,[551] although the gastrointestinal aspects are not.[547] Acute HDT bed rest has been shown to consistently induce increases in both intraocular (IOP) pressure[588] and ICP,[589] with the level of increase in both IOP and ICP appearing to be dependent on the angle of tilt imposed on the subjects.[524, 590, 591] A small (2-week) shuttle study did indicate an increase in inflight IOP.[592]

With specific respect to visual disturbances, space associated neuroocular syndrome (SANS) is an ocular condition now associated with long duration spaceflight and characterized by decreased near-visual acuity, globe flattening, optic nerve head elevation, cotton wool spots, unilateral and bilateral optic disc edema, choroidal thickening and retinal folds, hyperopic refractive error shifts, and nerve fiber layer infarcts.[8, 428, 499, 541, 565, 593] Of note, one study has correlated increases in lateral ventricle volume with optic disk edema and total retinal thickness, although no association was seen with changes in white matter or CSF volume.[538] Visual disturbances also have been identified in ground-based models. In short-term (<10 day) studies, Kermorgant *et al*. demonstrated that, during five days of dry immersion, there was enlargement of the optic nerve sheath diameter, although no change in IOP.[548] In medium-duration studies (≤30 days HDT bed rest) conducted under mild hypercapnic conditions, several ocular changes have

been identified: for example, Laurie *et al*. reported optic disk edema and quantified increases in peripapillary total retinal thickness in 5/11 subjects,[518, 519, 594] while others demonstrated progressive retinal thickening in the absence of optic disk edema, but with mild elevation of IOP.[549, 550] Longer (~60 days HDT bed rest) studies have described the development of choroidal folds as well as increased total retinal thickness, but with no change in visual function outcomes.[595] However, to our knowledge, other characteristics of SANS, such as changes in near-visual acuity, globe flattening, optic nerve head elevation, cotton wool spots and hyperopic shifts, have not yet been demonstrated using ground-based models.

We scored only a small number of space- and ground-based animal studies addressing neuroocular issues. Although there appears to be evidence that increased ICP is independently associated with visual issues in a non-microgravity murine model, such as optic nerve axonal loss and disorganization,[596, 597] we were unable to identify data indicating that increased ICP occurs in either rats or mice during spaceflight, although Mao *et al*. demonstrated a reduction in mouse IOP following a 35-day flight versus preflight.[362] The same study also showed significant apoptosis in the retina and retinal endothelial cells, which the authors suggested indicated decrements in the integrity of the blood-retina barrier,[362] a hypothesis supported by proteomic analysis.[557] A time-dependent increase in apoptotic cells in the retinal endothelium also has been seen in HLU mice over a 30-day period.[562] In addition, dystrophy of photoreceptor rods and cones was seen, more directly indicative of potential visual impairment.[558]

5.2.1.3. *Sensorimotor/Cognitive*: Effective decision-making is a critical component of manned spaceflight, and research suggests that stressors, such as hypercapnia[517] and space radiation,[598] may affect cognition and executive performance.[599] Although, currently, there is little evidence to support (or refute) space-induced effects on cognitive performance,[494] it seems likely that microgravity generates an environment where sensorineural functions are continuously challenged due to the loss of graviception.[600] Therefore, it appears necessary to determine the effects of microgravity on brain function, behavior and health.[601, 602]

It is evident that astronauts undergo a period of adaptation in order to control posture, eye-hand coordination, spatial orientation and navigation, not only when entering and during weightless conditions,[432, 505, 603] but also on their return to Earth.[505, 506, 603] Using visuospatial task performance as an index, Takács *et al*. demonstrated a gradual slowing in reaction time and a

decrease in accuracy relative to preflight across a 1.5—2 month mission period, suggesting an ongoing impact on cognitive performance.[508] Similarly, Moore *et al*. showed that, on the day of return following a 6-month spaceflight, astronauts exhibited significant deficits in manual dexterity, dual-tasking and motion perception.[604] Although these deficits may be a consequence of the changes in brain structure described above,[539] Cebolla *et al*. and others have suggested that alterations in the perceptual weighting given to visual cues under low gravitational conditions might result in greater demands on, and shifts in brain activity to, the motor cortex, cerebellum and vestibular network,[506, 603, 605] with progressive decrements in visuospatial performance.[508] Interestingly, although Tays *et al*. demonstrated significant declines in sensorimotor tasks pre- versus postflight, they saw no change in cognitive behavior.[507] Overall, it appears that few declines in basic cognitive functions in space have been observed, although deficits in perceptual-motor functioning[495] and divided attention due to cognitive overload[606, 607] have been identified.

The brain also appears to undergo a physiological adaptation during ground-based studies. Differential changes in brain activity have been seen,[608] although results have been heterogeneous, including an inconsistent response to the presence or absence of hypercapnia.[608, 609] For example, MRI during seven days of HDT bed rest demonstrated differential changes in activity in the posterior cingulate cortex (increase) versus the anterior cingulate cortex (decrease), proposed as part of an adaptation process since these areas are involved in internally-directed cognition.[610] Salazar *et al*. also suggested that, during early (HDT days 1—7) visuomotor adaptation, there was decreased brain activation in the temporal and subcortical regions, whereas during late adaptation (HDT day 29), there was increased activation in the right fusiform gyrus and right caudate nucleus.[518] In contrast, Mahadevan *et al*. saw early decreased activity in the superior frontal gyrus, although this was transient and seen only under hypercapnic conditions.[609] Over a long-term 70-day course of HDT bed rest, Yuan *et al*. described a gradual increase in the bilateral insular cortex, and suggested that this was a response to the reduction in somatosensory input experienced during bed rest.[552] Interestingly, a number of investigators also have identified rapid changes in cognitive function during the acute phase of HDT bed rest. For example, only three hours of HDT bed rest eliminated startle reflex plasticity compared to seated controls,[611] and, in another small study, investigators demonstrated a significant decrease in cognitive speed during short-term (26 hrs) – 12º HDT; surprisingly, this latter effect was reversed by simultaneous exposure to 0.5% CO_2.[520] Basner *et al*. also showed steady changes across a range of cognitive domains for the first 30 days

of HDT bed rest, although these were followed by a plateau from day 30 to 60.[523] However, a 30-day HDT bed rest study failed to identify any changes in dual task performance, assessed by reaction time and accuracy, with a small improvement in accuracy seen in the normal versus hypercapnic group.[609]

In one of the few behavioral studies performed on rodents flown in space, Ronca *et al*. demonstrated that younger (16-week), though not older (32-week), mice developed a coordinated group activity suggestive of a stereotyped motor behavior.[527] Although an interesting observation, there was little discussion regarding the age-specificity seen in the response, a significant omission given that the older animals were more age-equivalent to the astronaut cohort. Of the ground-based animal studies scored for this report, 7—28 days of HLU in rats progressively affected learning and memory,[534, 612, 613] which the investigators linked to disruption of hippocampal neuro-transmitter expression,[612] particularly acetylcholine.[534, 613] A more complete simulation of the complex space environment, including microgravity, social isolation, noise and altered circadian rhythms, also demonstrated decrements in learning and memory,[614] although an insufficient number of control groups were included to enable the reader to isolate effects from any individual stressor.

5.2.2. *Time Line of Progression*

5.2.2.1. *Structural Changes*: Due to the propensity of spaceflight studies using pre- versus postflight data only (21/25 of the scored CNS studies), the initiation time point of the various effects and their progression inflight is largely unknown. Based on postflight data, the incidence rate of many of the structural changes appears to be mission length-dependent, including the narrowing of the central sulcus (seen in 17/18 astronauts after long duration missions versus 3/16 after short duration),[433] upward shifting of the brain (12/18 astronauts versus 6/16, respectively), and narrowing of CSF spaces at the vertex (12/18 vs. 1/6, respectively).[433] Of the scored human ground-based publications, Marshall-Goebel *et al*. were alone in demonstrating an early increase in CSF volume, occurring within five hours of beginning HDT bed rest, with the magnitude of effect being tilt angle-dependent.[545]

Little information is available on the time line for CNS effects from animal flight studies since only two of the scored publications performed assessments inflight,[527, 554] and only one addressed structural changes, looking at animals on the last day of flight.[554] In that study, values

were compared to age-matched ground-control cohorts and showed that there was reorganization of the endothelial cells in the rat choroid plexus after 12 days of flight.[554] Although Mao *et al*. also saw increased hippocampal apoptosis and disturbance in blood-brain barrier integrity following a 35 day flight, suggestive of neurovascular damage, this was determined after ~38 hours of recovery with no additional time points or pathological confirmation.[333] The scored ground-based animal studies were similarly limited to an assessment of effects at the termination of HLU. Chen *et al*. demonstrated that 21, but not seven, days of unloading induced regional neuronal atrophy in three out of six rat cerebral cortices.[576] Other investigations addressed relative changes in hippocampal neuronal firing rate,[533] as well as neurotransmitter,[578, 612] mitochondrial ROS,[532] gene[492, 561] and protein[577] expression induced by unloading; although, by implication, the observed signal changes likely reflect cytomorphological alterations, these were not confirmed pathologically.

5.2.2.2. *Neurovestibular/Neuroocular (SANS)*: Neurovestibular disruption begins within minutes to hours of achieving weightlessness, with ~70% of astronauts rapidly experiencing space motion sickness symptoms.[580] However, resolution is equally quick, with symptoms diminishing within 8—72 hours.[615] The relatively high incidence of neuroocular effects in astronauts has led to well-defined incidence rates of the associated optical effects,[540] with evidence of mission length-dependence. For example, in a review by Lee *et al*., the authors describe findings from a survey of 300 astronauts showing that short-duration shuttle crews reported 7% and 23% inflight decreases in distant versus near visual acuity, respectively,[499] compared to 12% and 48% decreases in long-duration flight members.[499] With respect to other characteristics of SANS seen postflight, in a group of 27 astronauts, Kramer *et al*. demonstrated posterior globe flattening in 26%, optic nerve protrusion in 15%, moderate concavity of the pituitary dome with posterior stalk deviation in 11%, and a central area of T2 hyperintensity in 96%, as well as an increase in optic nerve diameter that occurred in association with kinking of the optic nerve sheath.[511] However, the time lines of initiation and progression of the various changes are unknown, and the incidence of these effects can vary among missions and crews; for example, optic disk edema was seen after long-duration flights in 3/18 astronauts in one study,[433] but in only one of ten in another.[510]

With respect to the incidence rates and time lines of neurovestibular/neuroocular effects in human ground-based models, van Oosterhout *et al*. have catalogued the incidence of headaches during the course of five days of HDT bed rest, showing that 64% of participants experienced a

headache at some point, with the majority occurring within the first day.[546] In addition, neurovestibular symptoms consistent with SMS have been exhibited within the first day of HDT bed rest,[551] although gastrointestinal symptoms have not been experienced.[547] In terms of specific visual disturbances, a short-term study of one hour of HDT bed rest failed to induce any significant change in IOP,[521] whereas Taibbi *et al*. demonstrated an acute increase in IOP on entering HDT, which stabilized across the study period.[550] Interestingly, immediate changes in IOP have been seen in a rabbit study, with increases in IOP being tilt angle-dependent.[616] Kermorgant *et al*. demonstrated an 11% increase in optic nerve sheath diameter on day one of dry immersion, with a progressive increase over the five day period.[548] The more commonly observed symptom associated with the SANS spectrum, *i.e*. optic disk edema, has generally been observed only at the end of medium-term (~30 days) bed rest.[396, 594] However, Taibbi *et al*. compared optical changes following short-term (14 days) versus long-term (70 days) HDT bed rest and showed that, of the spectrum of effects induced by short-term HDT bed rest, only peripapillary retinal thickening progressed[549] and occurred in the absence of optic disk edema.[550]

None of the scored animal flight studies performed inflight analyses with respect to neuroocular events; animals were assessed within hours (3—48 hours) postflight. Mao *et al*. saw increased retinal apoptosis in mice following 13 and 35 days of flight, respectively,[557, 559] which persisted from 3—5 hours to two days relative to ground-based controls, with increased apoptosis seen in the retinal vascular endothelium.[362] Furthermore, following 35 days flight, additional retinal changes were seen at 28 hours postflight, including significant decreases in the thickness of total retina, retinal pigment epithelium, and choroid layers, with choroid deformation and folds also being observed.[558] Increases in retinal apoptosis also have been seen following HLU, observed at both four and 30 days after reambulation, with levels exacerbated when HLU was combined with a single exposure to protons.[562]

5.2.2.3. *Sensorimotor/Cognitive*: In the scored publications, limited cognitive testing was performed on astronauts inflight. Harris *et al*. showed that visual perception of orientation in astronauts was unaltered early in flight (day two),[505] although Cebolla *et al*. suggested that shifting in brain activity towards the motor cortex occurred around flight day nine and persisted until late in flight (flight day ~55).[506] Similarly, Takács *et al*. suggested that cognitive impairment was present by flight day eight, and progressed through flight day ~50,[508] although Tays *et al*. saw no

change in cognitive performance between flight days 30—180.[507] In contrast, ground-based human models have demonstrated a rapid induction of cognitive and sensorineural effects. For example, in a small, short-term study, Basner *et al*. demonstrated a progressive increase in response speed over a 0.1—21 hour period of 12º HDT bed rest using a Cognition Test Battery, with a concomitant loss of accuracy,[520] and Messerotti Benvenuti *et al*. saw a loss in startle reflex by hour three.[611] Liao *et al*. showed shifts in brain activity by day two of HDT bed rest,[610] whilst others saw changes in visuomotor performance by days 7—9 of HDT bed rest, with either a plateauing or worsening in effect up to, and beyond, day 21.[517-519, 552]

As noted in the previous paragraphs, few data are available from the scored animal studies. One investigation performed regular inflight behavioral testing, during which time, animals were seen to begin stereotypical motion on day ~8—10 of flight.[527] Although Kwok *et al*. demonstrated that 12 of 18 flown mice had significantly altered gait characteristics following 35 days of flight, likely reflecting sensorimotor adaptation during weightlessness,[560] neither inflight nor additional postflight time points were assessed. In general, the scored ground-based animal studies also supported a rapid onset and persistence of cognitive effects. For example, Wang *et al*. demonstrated deficits in spatial learning and memory within 1—2 days of HLU,[533] although Zhang *et al*. suggested these were unaffected until >seven days of HLU;[534] Qiong *et al*. showed a decline after 14 days of HLU.[613]

5.2.3. <u>*Recovery Kinetics*</u>

5.2.3.1. *Structural Changes*: Of the six scored astronaut studies that performed multiple postflight analyses, Kramer *et al*. followed a cohort over a recovery period of one year, using MRI to assess intracranial changes.[509] The team showed that, not only was the summated mean brain (white plus gray matter) and CSF volumes increased on recovery day one relative to preflight, but that this increase persisted until 1-year postflight. However, not all observed structural changes appear to persist: for example, a partial reversal of increased white matter hyperintensity volume and increased ventricular CSF volume has been seen by one month postflight.[537] In addition, long-term follow up (~200 days postflight) demonstrated partial recovery of gray matter volume towards preflight levels in most areas of the brain,[431] a global reduction in cerebral white matter volume,[431] and a return to preflight levels in ventricular and CSF volumes.[6, 9, 431] Few of the scored ground-based human studies performed structural analyses during the reambulation period, and none were

taken at recovery time points beyond two weeks. Nonetheless, of the regional-specific changes seen in gray matter following 30 days of HDT bed rest, such as increased gray matter volume in the posterior aspect of the vertex, decreased volume at the base of the cerebrum and increased free water redistribution,[396] none had recovered to baseline by day 12 of reambulation.[396, 573]

Scored studies assessing CNS structural recovery in animals were limited. Of those, the changes in apical organization observed in the choroid plexuses of rats following a 9-day spaceflight appeared partially complete by day two of recovery,[553] whereas, following 21 days of HLU, the cortex and hippocampus of mice exhibited multiple, albeit differential, changes with respect to microvascular density and tortuosity, both at seven days and persisting until nine months of reambulation.[59]

5.2.3.2. *Neurovascular/Neuroocular (SANS)*: Likely due to our current medical abilities to restore or correct for visual disturbances, we identified few studies that addressed downstream postflight consequences in the neurovascular/neuroocular field. With respect to neuroocular alterations, some residual refractive errors have been shown to persist following spaceflight for as long as several years postflight,[499] although, again, neither incidence nor persistence was confirmed in all studies.[510] The increase in IOP seen inflight in a 2-week Shuttle study returned to baseline by recovery day 35,[592] whereas in the scored bed rest studies, the acute increase in IOP seen at the onset of HDT had normalized by day nine of reambulation;[550] no change in IOP, either during or after dry immersion, has been described.[548]

5.2.3.3. *Sensorimotor/Cognitive*: The sensorimotor adaptations that occur in the space environment become inappropriate on landing, leading to astronaut postural and gait difficulties and balance deficits seen over the first week of the recovery period,[544, 617] with some suggesting that the reweighting of perceptual cues used in postural orientation may take even longer to recover.[505] Interestingly, one study showed that those astronauts demonstrating vestibular-driven balance deficits on landing also exhibited skin hypersensitivity, consequently proposed as a potential biomarker for postural issues,[542] although the physiologic link was undetermined. Assessment of sensorimotor function using balance tests has indicated a mission duration-dependent level of decrement and recovery period: short-flight subjects exhibit degraded performance immediately on return, but with the majority of parameters recovering by day one; in

contrast, long-flight subjects show more significant decrements in performance on recovery day one.[256, 507] Furthermore, although Mulavara *et al*. suggested almost complete recovery of sensorimotor function by day six postflight,[256] Tays *et al*. noted only partial recovery by day 30, although with continued improvement over the subsequent 60 days.[507] Determination of cognitive deficits and their recovery time line is unclear and heterogeneous: Tays *et al*. saw no change in cognitive performance following long-term missions;[507] decrements in cognitive performance were seen by Takács *et al*. during early recovery (recovery days 2—8), but with a return to baseline by days 15—22;[508] others have provided evidence of postflight cognitive decline in performance persisting for 6-months in both speed and accuracy domains.[428] In human ground-based studies, Mulvara *et al*. demonstrated that both functional and clinical tests of balance, used as a means of assessing the sensorimotor system, remained significantly affected for 6—12 days of recovery following 70 days of HDT bed rest.[256] However, Basner *et al*. showed that cognitive decrements, seen across multiple cognitive domains during 30 or 60 days of HDT bed rest, were completely recovered by day 15 following reambulation.[523]

With respect to the animal models, interestingly, Temple *et al*. assessed cognitive spatial learning and memory in rats that had been flown for 16 days during development, *i.e*. as pups. They saw only subtle differences between the behavior of flown versus ground-control animals, with any differences normalizing rapidly within the first few days.[526] Of the few other scored ground-based animal studies that addressed long-term endpoints, none described recovery *per se*, but instead assessed the development of late decrements during the delayed to late reambulation periods.[528, 529] In one study, assessments were performed at nine months following 30 days of HLU, with cognitive testing of rats indicating an impairment in spatial habituation learning.[528] In a second study, mice underwent cognitive testing at one week, one, four and eight months following three weeks of HLU.[529] The animals exhibited abnormal exploration and/or high-risk taking behavior; however, it appears that data were pooled for all time points, so that temporal changes across the recovery period were not discernible.

5.2.4. *Potential Mechanisms*

As with the previous sections, the most frequently hypothesized induction mechanism underlying many of the CNS changes seen in astronauts is a microgravity-induced fluid shift, specifically towards the head, leading to changes in intracranial pressure (ICP).[497, 618] This phenomenon has

been proposed as responsible for the changes in blood-brain barrier integrity,[333, 529] venous blood flow,[16] and increased ocular pressure[9, 582, 619-621] observed in astronauts, as well as the various microgravity models. This concept is supported by the general characteristics of SMS, which, in terms of symptoms and time line, resembles benign intracranial hypertension;[622] other factors that might contribute to increased ICP include venous outflow obstruction and disruption to CSF flow.[582] However, we are unaware of any study confirming inflight changes in astronaut ICP. Mader *et al*. also proposed that the ocular and optic nerve changes seen as part of SANS are directly induced by cephalad fluid shifts.[540] Interestingly, SANS was initially entitled as the Visual Impairment Intracranial Pressure syndrome since it was thought that increased ICP was the critical, if not singular, underlying mechanism; however, a growing appreciation of other potential contributing factors, such as cephalad fluid shifts, venous/lymphatic stasis, inflammation, *etc*., have led to the redesignation as SANS.[8] Currently, the two chief hypotheses proposed for SANS involve increased ICP due to cephalad fluid shifts[618] versus compartmentalization of CSF to the globe,[623] two concepts that are not mutually exclusive[565] since they may be linked through the interaction of multiple factors.[624]

In addition to their involvement in the development of SANS, the loss of hydrostatic pressure and altered hemodynamics in the intracranial circulation and CSF system likely elicit adaptations of multiple structures and fluid systems within the skull.[586] However, interrelationships between the observed effects and the plethora of potential triggering and/or contributing factors remains unclear given the current level of data, compounded by the heterogeneity in findings. For example, Alperin *et al*. showed significant pre-to-postflight increases in globe flattening and optic nerve diameter, major characteristics of SANS, and described an association with significant increases in orbital and ventricular CSF volumes,[619] yet Roberts *et al*. identified astronauts with SANS as having relatively smaller changes in ventricular volume compared to those that did not.[539] Without astronaut cohorts of sufficient size and standardized testing across systematically acquired and rational time points, the mechanisms underlying such a diverse range of CNS effects remains, at present, hypothetical.[494]

The potential role for fluid shifts and/or changes in cephalad-related pressures as a mechanism underlying astronaut CNS effects is consistent with those proposed in human ground-based models, in particular with respect to optical changes. For example, increases seen in orbital (and possibly extracranial) CSF pressure have been associated with increasing optic nerve

diameter.[545] Similarly, changes in free water distribution in the optic nerve, together with increased CSF volume and movement within the optic nerve sheath, have been proposed as resulting from perioptic CSF hydrodynamics during HDT bed rest.[522] However, despite their associations with optical effects, no structural or fluid brain changes have correlated with cognitive performance. For example, functional MRI following 45 days of HDT bed rest has resulted in decreased degree centrality in the left anterior insula and the dorsal anterior cingulate,[625] suggesting that HDT bed rest was specifically affected by the salience network. This region is seen as the most consistently affected in both space- and ground-based studies,[517] and exhibits decreases in functional connectivity, a possible reflection of adaptation due to the provision of incongruent information. Indeed, maladaptation and or readaptation, especially in the context of spatial and positional referencing, has been highlighted as a potential mechanism in a number of the visuo- and sensorimotor issues experienced during, and more especially following, spaceflight.[600, 605]

Animal studies seem unlikely at present to cast any additional light on the role of fluid shifts in CNS effects. Indeed, some of the fluid shifts that are seen in humans in both space and HDT bed rest are not recapitulated in rodents, likely due to their small body size.[626] Although not necessarily related, the mechanistic focus of most animal studies has been, as described previously, at a more cellular level. For example, Mao *et al*. have described increased levels of apoptosis in regional brain areas, and especially in vascular endothelial cells, suggesting that one mechanism underlying CNS effects may be neurovascular damage associated with the loss of blood-brain barrier integrity.[333, 362, 557, 559] Similar decrements in the blood-retinal barrier also may offer a possible explanation for changes in visual acuity and impairment.[558] In addition, a review of neurotransmitter networks determined from rodents flown in space has suggested that changes in, for example, the dopaminergic system may provide an explanation for the dysfunction seen in both movement and behavior in astronauts.[627] However, the effects of long-term spaceflight seen in both the serotonin and dopaminergic systems of rodents, as well as changes in some apoptotic factors, have not been recapitulated consistently across HLU studies.[628]

6. Immune Effects

In a 2010 review of the influence of microgravity on overall astronaut health,[155] the functionality of the immune system was described as a major concern for spaceflight given its critical role in host protection and the potential health risks associated with deep space exploration-class missions.[629]

6.1. Publication Scores and Characteristics

6.1.1. *Astronaut Category* (**Table 13**)

- Of the 21 studies scored in this category, all but one[630] had mixed gender groups, although none considered sex as a factor despite acknowledged sex-dependent differences in immune responses.[631, 632] Nonetheless, it is recognized that, of the combined total of ~320 subjects included in the scored studies, 264 (82%) were male, reflecting the overall gender bias in the astronaut workforce.
- Unusually for the astronaut studies scored for this report, nearly half of the studies made use of a ground control group,[633-642] 11 had taken inflight samples[630, 633, 635, 638, 639, 642-647] and ten had looked at time points beyond the first few weeks of recovery.[630, 635, 638, 639, 642-644, 647-649]

6.1.2. *Ground-Based Human Category* (**Table 14**)

- In the ten studies scored in this category, few of the additional physical factors associated with the space environment were included in the experimental design: one study[650] described the development of a HDT model that included a simulation of the accelerated forces experienced during launch and re-entry using centrifugation, and only three studies controlled the diet of participants.[650-652]
- The majority of the 368 subjects were male (92%), with only one study using a mixed-gender group[650] and one using females only.[652]
- Only two studies looked beyond the first two weeks of recovery,[652, 653] although all performed intermediate analyses during HDT.

6.1.3. *Animals In Space Category* (**Table 15**)

- All of the 24 publications considered in this category made use of rodent models, predominantly mice (9:15, rat:mouse), and all of the latter on a C57BL/6 background.
- All studies used single gender (12:12, male:female), with only two studies using fully age-appropriate animals,[654, 655] and 14 looking at juveniles.
- All studies employed a matched ground-control group, with the majority including an additional matched vivarium cohort; only two of the studies looked beyond an acute (within hours) recovery time point.[656, 657]

6.1.4. *Ground-Based Animal Category* (**Table 16**)

- All but two (rat)[658, 659] of the 26 studies considered in this category used murine models; two of the studies utilized PWB.[660, 661] Interestingly, a range of mouse strains were used in the studies, with 5/26 using the ICR outbred strain whereas 11/26 used the inbred C57BL/6 strain.
- Eight of the studies used a combined HLU-radiation model;[57, 662-668] one of the PWB studies delivered the radiation prior to unloading.[661]
- Three of the 26 studies scored in this category used both male and female subjects,[663, 669, 670] with one considering sex as a factor.[663] One of the studies used age-appropriate animals,[666] three had used young (4-months old) adults,[667, 671, 672] with the remainder of the studies using juveniles.
- 7/26 of the studies had performed intermediate analyses during unloading, although only three studies made use of time points beyond the immediate point of removal from suspension.[649, 666, 673]

Table 13: Highest scored publications in the astronaut-immune category.

ASTRONAUT - IMMUNE	Environment parameters	Astronaut characteristics	Science / time points	Score	Main Scientific Findings
Short flight (<30 days)					
Mehta SK *et al*. 2014[633]	9	3	9	21	12—16 days flight: Reactivation and shedding of several latent herpes viruses seen in 14/17 astronauts
Pierson DL *et al*. 2005[638]	9	3	9	21	5—14 days flight: Significant increase in shedding of EBV† during flight
Mehta SK *et al*. 2013[639]	9	3	9	21	9—14 days flight: Association between virus shedding and elevated levels of specific plasma cytokines
Kaur I *et al*. 2008[640]	9	3	6	18	5—11 days flight: Response of monocytes to gram-negative endotoxins modified during and postflight
Crucian B *et al*. 2013[645]	9	3	5	17	10—15 days flight: Leukocyte distribution, T cell function and cytokine expression modified inflight
Kaur I *et al*. 2005[634]	9	3	5	17	5—11 days flight: Reduced phagocytotic function in monocytes, associate with changes in CD32 and CD64 expression
Long flight (≥4 months)					
Agha NH *et al*. 2020[647]	9	3	9	21	>6 months: Early release of salivary antimicrobial proteins; shedders also release stress markers
Bigley AB *et al*. 2019[643]	9	3	8	20	>100 days flight: NK†† cell function reduced; higher in "rookies" versus experienced astronauts
Mehta SK *et al*. 2017[635]	9	3	8	20	~180 days flight: 22/23 astronauts shed herpes virus, with 8/23 long-term astronauts shedding multiple (EBV, VZV†, CMV†); viruses reactivated independently
Spielmann G *et al*. 2019[642]	9	3	8	20	6 months flight: B cell homeostasis maintained throughout flight
Crucian B *et al*. 2015[644]	9	3	7	19	6 months flight: Persistent reduction in T cell (CD4 and CD8) function across mission: elevated WBC††† count; reduced production of mitogen-induced cytokines
Urbaniak C *et al*. 2020[630]	9	1	9	19	2—9 months flight: Significant changes in salivary microbiome during flight; possible correlation with virus reactivation
Stowe RP *et al*. 2001[636]	9	3	5	17	~180 days flight: Elevated levels of EBV-specific antibodies pre- and significantly elevated postflight. Proposed preflight values were associated with chronic stress; additional inflight events triggered replication

Benjamin CL *et al*. 2016[648]	9	3	4	16	~184 days flight: Suppression of thympoiesis seen post-flight. Possible effect on T cell repertoire
Agha NH *et al*. 2020[637]	9	3	4	16	>100 days flight: Reactivation of EBV, VZV and CMV seen pre-, during, and postflight. Levels mitigated with increased CRF*

† EBV: Epstein-Barr virus; VZV: varicella-zoster virus; CMV: cytomegalovirus
†† NK: natural killer cells
††† WBC: white blood cell (leukocyte)
* CRF: cardiorespiratory fitness

Table 14: Highest scored publications in the ground-based human-immune category.

HUMAN BED REST STUDIES – IMMUNE	Environment parameters	Astronaut characteristics	Science / time points	Score	Main Scientific Findings
Short-term (<5 days HDT)					
Feuerecker M *et al*. 2013[674]	2	2	5	9	5 days HDT (+/- artificial gravity): Shedding of CD62L (L-selectin) appeared non-inflammatory, possibly due to head-down fluid shifts
Medium-term (10—30 days HDT)					
Stowe RP *et al*. 2008[650]	4	3	7	14	16 days HDT (+launch/re-entry simulation): Changes mirrored 9-day (not 16-day) Shuttle flights: persistent elevation urine cortisol; differential changes in monocyte/eosinophil numbers
Kelsen J *et al*. 2012[651]	3	2	5	10	21 days HDT: Decrease in mitogen-induced cytokines (IL-2, IFN-γ, TNFα†); no virus reactivation
Long-term (≥50 days HDT)					
Schmitt DA *et al.* 2000[653]	2	2	7	11	42 days HDT: T cell/monocyte numbers unaffected; TNFα secretion did not change; other mitogen-induced cytokines increased transiently
Shearer WT *et al.* 2009[652]	3	2	4	9	60 days HDT (+/-exercise) + bacteriophage immunization: Increased TNFα levels. Exercise accelerated antibody production and mitigated TNFα
Bonnefoy J *et al*. 2022[675]	2	2	5	9	60 days HDT: No effect on B cell homeostasis

† IL-2: interleukin-2; IFN-γ: interferon-γ; TNF-α: tumor necrosis factor-α

Table 15: Highest scored publications in the space animal-immune category.

ANIMAL-SPACE STUDIES – IMMUNE	Environment parameters	Astronaut characteristics	Science / time points	Score	Main Scientific Findings
Short flight (~30 days)					
Novoselova EG *et al*. 2015[657]	8	2	5	15	30 days flight: Reduced splenic/thymic masses and associated lymphocytes. IL-6 and IFN-γ (not TNF-α)† levels reduced
Tascher G *et al*. 2019[654]	8	3	4	15	30 days flight: 1-week postflight, adverse effect on B-cell lymphopoiesis with 41% reduction in splenic B cells
Sonnenfeld G *et al*. 1998[676]	6	2	5	13	11 days flight: Immune effects in flown pregnant dams not seen in pups, born postflight
Gridley DS *et al*. 2009[677]	7	0	5	12	13 days flight: CD3+ T cells and CD19+ B cells reduced; NK cells†† increased. Altered T cell distribution, function and gene expression seen immediately postflight
Nash PV *et al*. 1992[678]	6	2	4	12	13 days flight: No effect seen on lymphocyte proliferation or cytokine (IL-2) expression in rat peripheral lymph node (inguinal) tissue
Pecaut MJ *et al*. 2003[679]	7	0	5	12	12 days flight: Shift in splenic lymphocytes from T cells to B cells. Suggested shift in bone marrow populations
Ward C *et al*. 2018[655]	6	3	3	12	Sacrificed 21—22 days flight: Inflight samples indicated no effect on B cell repertoire
Baqai FP *et al*. 2009[680]	7	0	5	12	13 days flight: Reduction in liver, spleen and thymic mass; lymphocytes, monocyte/macrophages, granulocyte counts reduced. Secretion of IL-6 and -10 increased (not TNF-α)
Gridley DS *et al*. 2003[681]	7	0	5	12	12 days flight: Decrease in thymus and spleen size; increased numbers of T cells and NK cells
Lesnyak A *et al*. 1996[656]	6	1	5	12	14 days flight: T cell and NK cell activity decreased on landing; normalized by R14 days
Ortega MT *et al*. 2009[682]	7	0	5	12	13 days flight: Shifts in bone marrow phenotype/ differentiation suggest altered macrophage populations
Grove DS *et al*. 1995[683]	7	0	5	12	10 days flight: Altered activation potential among splenocytes and lymph node lymphocytes, as well as redistribution among the organs
Long flight (>30 days)					
McCarville JL *et al*. 2013[684]	9	0	3	13	91 days flight: Reduced levels of IL-2 and TGFß††† (not seen in transgenic PTN* mice)

† IL-6: interleukin-6; IFN-γ: interferon-γ; TNF-α: tumor necrosis factor-α
†† NK: natural killer cells
††† TGFß: transforming growth factor-ß
* PTN: pleiotrophin

Table 16: Highest scored publications in the ground-based animal-immune category.

GROUND-BASED ANIMAL STUDIES – IMMUNE	Environment parameters	Astronaut characteristics	Science / time points	Score	Main Scientific Findings
Short-term (≤10 days)					
Li M *et al*. 2014[663]	5	1	4	10	10 days HLU +/- gamma/proton (SPE†): Exposure to bacterial infection (*Pseudomonas aeruginosa/ Klebsiella pneumoniae*) showed increased mortality/ decreased clearance, greatest in combined stress group
Li M *et al*. 2015[57]	5	0	5	10	10 days HLU + γ/proton +/- G-CSF†† or enrofloxacin: Mortality from exposure to *Pseudomonas aeruginosa* in combined stressor group reduced by both counter-measures
Zhou Y *et al*. 2012[664]	5	0	4	9	9 days HLU + γ/proton: Combined stress group showed acute systemic immune activation (loss of LPS containment); resolved <7 days post-radiation
Medium-term (10—30 days)					
Paul AM *et al*. 2021[666]	5	3	3	11	21 days HLU +/- LD/DR γ-radiation: At 7 days post-HLU, little difference in immune differentials, but altered RBC* morphology, dysregulated immune and inflammation profiles
Paul AM *et al*. 2020[671]	4	2	3	9	14 or 30 days HLU: Increased neutrophil-to-lymphocyte (NLR) ratio corresponded to OS***-driven inflammation – potential biomarker
Takahashi A *et al*. 2018[685]	4	0	5	9	24 days HLU: Unloading significantly increased tumor growth, lung metastasis and splenic/thymic atrophy

† SPE: solar particle event
†† G-CSF: granulocyte-colony stimulating factor
††† LD/DR: low dose/dose rate
* WBC/RBC: white blood cell (leukocyte)/red blood cell
** GCR: galactic cosmic ray simulation
*** OS: oxidative stress

6.2. Immune Microgravity Studies: Data Comparisons Across Models

Analysis of blood samples from returning astronauts on early missions raised concerns due to a decline in T cell responsiveness,[686] with suggestions that the immune system was depressed in more than 50% of the astronauts, both during and after spaceflight.[687] Despite a high level of variability among studies and models,[688, 689] data have persistently demonstrated significant and differential effects in various elements of the immune system,[690] including the regulatory cytokine network,[691, 692] indicating that the astronaut immune system undergoes profound changes as a consequence of spaceflight, with the potential to affect responses to infection and wounds, as well as allowing for virus reactivation.[693] Of note, some current hypotheses suggest that these and related observations are the consequence of a chronic stress response, rather than an adaptation to microgravity *per se*,[694, 695] with the observed immune alterations being due to a combination of both physical and psychological stressors.[696, 697]

6.2.1. *Immune-Associated Outcomes*

Spaceflight studies have indicated that astronauts undergo significant immune dysregulation and alteration, with reports of a variety of immune cells changing phenotypically and functionally.[697] For example, assessing the effects of short-duration (10—15 day) flights, Crucian *et al*. observed significant changes among specific subsets of CD8+ T cells assessed inflight one day prior to landing, although no alterations in white blood cells (WBC) or absolute levels of leukocyte or lymphocyte subsets were observed at the time.[645] Strikingly, the absolute level of cytotoxic CD8+ cells increased prior to, as well as during, spaceflight, although true naïve, terminally differentiated and senescent T cells decreased inflight only, consistent with changes that were subsequently seen to persist across longer (6-month) missions.[644] Furthermore, the same group performed sequential sampling across a 6-month flight study and saw early (~flight day 14) and persistent (flight days 2—4 months, 6-months) increases in both WBC and granulocyte numbers;[644] the group also confirmed a decrease in T cell function, as well as an increase in natural killer (NK) cell numbers during the late flight period (~six months). Interestingly, investigators have demonstrated that the killing efficiency of astronaut NK cells decreases during both acutely (21 days)[698] and progressively across a 6-month mission,[643] with the latter study showing greater decrements in rookie crew members versus veteran fliers.[643] However, with respect to humoral immunity, Spielmann *et al*. saw no changes in the number of total B cells (counts or proportion),

naïve/transitional or regulatory B cells during a 6-month spaceflight, an observation confirmed by Crucian *et al*.,[644] together with a modest trend towards an increased number of memory B cells.[642]

As described by Rooney *et al*., herpesviruses, in particular Epstein-Barr virus (EBV), varicella-zoster virus (VZV), herpes simplex viruses 1 and 2 (HSV-1 and -2) and cytomegalovirus (CMV), have evolved alongside humans for millennia; in immunocompetent people, such viruses will lie dormant following infection, but can become reactivated during periods of stress, immune challenge, *etc*.[697] However, although most astronauts, like the general population, are already infected with one or more latent herpesviruses, multiple studies now have demonstrated increased virus shedding in astronauts pre- and/or during flight relative to ground controls, including EBV,[633, 635-638] VZV,[633, 635, 637, 646] and CMV.[633, 635, 637, 699] In general, the shedding of live/infectious virus has been observed in the absence of disease,[646] although there has been at least one inflight case of HSV-1-associated dermatitis.[700] Stress hormones, generally assessed through salivary or urinary cortisol levels, also have been shown to be elevated pre- and/or during and/or postflight,[633, 635, 636, 638, 647] and some investigators have described a Th1—Th2 shift in plasma cytokine expression,[639] a potential biomarker for dysfunctional immunoregulation.

Using the human ground-based model, some immune effects have been demonstrated: for example, a short-duration (seven days) dry immersion study showed the induction of several negative shifts in the immune system, although the investigators pointed to the considerable heterogeneity in response;[701] a long-duration (42 day) HDT bed rest study saw significant increases in polymorphonuclear neutrophils (PMN),[653] and two long-duration (60 and 120 day) HDT bed rest studies demonstrated modest alterations in lymphocyte subsets.[702, 703] Interestingly, a horizontal bed rest study, in which additional treatment with hydrocortisone was used to simulate the stress associated with launch and reentry, led to increased shedding of EBV into the saliva,[704] and a study that used pre- and post-centrifugation as a simulation for launch and reentry in combination with 16 days of HDT bed rest also elicited changes in leukocyte and lymphocyte subsets and increases in urinary cortisol, which the authors believed more closely recapitulated some of the data from short-term flight missions.[650] However, in general, the majority of scored short-duration (5—21 days)[651, 674, 705, 706] and long-duration (42—90 days)[653, 707] HDT bed rest studies have failed to demonstrate any significant immune system alterations, although several studies suggested that shifts occurred in immune-related cytokine expression.[651, 652, 675, 708-710]

Of the limited number of scored inflight analyses performed on flown rodents, one study involved rats sacrificed during 14 days flight, and showed reduced T cell and NK cell activity with a reduction in immune-related cytokine expression.[656] Of the scored ground-based rodent studies that performed mid-suspension analyses and saw immune effects, Suzuki *et al*. showed an early change, with three hrs of HLU inducing a transient increase in lymphatic flow from the iliac lymph nodes, but with no change in lymphocyte subsets.[659] A number of investigations also reported early (day 2—4 HLU/PWB) increases in WBCs,[660, 662] lymphocytes,[660, 662, 665] and neutrophils,[662] but with a return to baseline levels by day seven of HLU, thus proposing observations as the result of a stress response to the HLU apparatus. Similarly, Zhou *et al*. reported rapid increases in levels of circulating LPS, suggestive of a transient increase in immune activation, that decreased with increasing periods (<nine days) of suspension.[664] Nonetheless, more prolonged immune effects have been seen: Romero-Weaver *et al*. saw a non-statistical increase in granulocytes at HLU day 6—18[665] and Paul *et al*. demonstrated progressively increasing numbers of circulating neutrophils during 14—30 days of HLU, but with no change in lymphocytes.[671] With respect to overall immune functionality in these studies, four days of HLU have been shown to suppress activation of splenic T lymphocytes,[662] and Li *et al*. demonstrated that five days of HLU modestly increased morbidity and mortality from a bacterial infection, with a significant additive effect due to combined exposure with an SPE-like irradiation.[57, 663] Other 14 day HLU studies have reported increased susceptibility to colonic inflammation with a decrease in Treg cell numbers[711] and suppression of cytokine-mediated intestinal immunity.[670] In addition, Rettig *et al*. showed that four weeks of HLU altered the T cell repertoire in response to challenge.[712]

6.2.2. *Immune-Associated Incidence and Time Line*

Sporadic testing and a lack of inflight sequential sampling limits the information available on the incidence and time line of astronaut immune effects. Indeed, it has been noted that prior to 1990, the majority of immune changes assessed during missions longer than two weeks were performed in cosmonauts, usually comparing pre- to postflight samples only.[713] As described previously, Crucian *et al*. observed changes among specific subsets of CD8+ T cells one day before landing after a 10—15 day flight,[645] specifically in true naïve, terminally differentiated and senescent T cells, with the increase subsequently shown to persist across longer-duration (6-month) missions.[644] Using early (flight day 14), mid- (2—4 months) and late (6-month) mission time

points, increased numbers of WBCs and granulocytes were seen early, (~flight day 14) progressively increasing with mission duration, and there was an increase in NK cell numbers during the late flight period (~six months),[644] although this was acknowledged as being at odds with findings from other studies.[698, 714] For example, Bigley *et al*. demonstrated that the killing efficiency of astronaut NK cells decreased progressively across a 6-month mission, although the absolute numbers of cells were unaltered.[643]

To date, and despite the considerable level of research conducted in the immune area, it does not appear that animal modeling, whether flown or ground-based, offers additional insight into the timing of space-induced immune changes, with many of the observed temporal changes chiefly being associated with transient apparatus-induced stress. The majority of the scored immune rodent ground-based studies performed analyses immediately at the end of suspension, thereby likely simulating conditions at the end of flight, *i.e*. prior to reentry. One of the most consistent observations has been the loss of thymic and/or splenic mass, seen as early as 48 hours following HLU,[715, 716] as well as following suspension periods ranging from three to 24 days.[669, 673, 685, 716] The observed atrophy, associated by some with increased levels of apoptosis in thymocytes and splenocytes,[669] may be cell type-specific, with reports of significant decreases in splenic B cells[715, 717, 718] and NK1.1+ cells.[715] However, a single 21-day HLU study reported no change in thymic or splenic weights, despite observing a significant decrease in splenic B cells, and with no change in T cells.[717]

6.2.3. *Immune-Associated Recovery Kinetics*

Decades of immediate (usually 3—4 hrs post-landing) and early postflight analyses of both cosmonauts and astronauts have highlighted significant alterations in the immune system, although the stress of reentry and rapid readaptation to gravity have been proposed as major contributors.[645] Examples of such observations include: a decline in cosmonaut immune function, assessed in terms of lymphocyte reactivity, T helper cell activity and NK cytotoxic capacity, effects that have lasted for 1—7 days following prolonged (3—11 months) space flights onboard Salyut 6, 7, and Mir;[719] reduced T lymphocyte reactivity in U.S. Space Shuttle astronauts seen on landing, in addition to an increase in neutrophils and a decrease in eosinophils.[686] Interestingly, even relatively minor differences in mission length appear to differentially affect some populations of immune cells; for example, although the majority of studies demonstrate significantly increased numbers

of WBCs, polymorphonuclear leukocytes and CD4+ T cells postflight, irrespective of mission length,[645, 696, 720] one study showed that, following a nine-day mission, monocytes were increased and NK cells were decreased, whereas after a 16-day mission, monocytes were decreased, but with no change in NK cells.[696] Crucian *et al*. confirmed an immediate post-landing increase in WBCs (absolute granulocytes),[645] as well as differential changes in T cell subsets[645] and a decrease in CD16+ monocytes, but saw no change in NK cells. All populations recovered to preflight levels by recovery day three,[720] although others have suggested that monocyte dysfunction persists for 6—12 months post-landing.[634, 640] By way of contrast, a small study of Chinese astronauts saw significant decreases in NK cell and reticulocyte numbers on landing following 13—15 days flight, with a return to baseline by recovery day ten.[649] Others also have shown that neutrophil samples from astronauts following a 5-day mission were equivalent in function to controls, whereas samples from those on 9—11 day missions exhibited increased numbers (85% increase compared to preflight) and reduced function on landing.[641] Furthermore, indicators of stress levels also have been shown to vary dependent on mission duration; for example, plasma cortisol levels were significantly decreased following 9-day missions, but increased after 16-day missions.[696]

Increased viral shedding by astronauts relative to controls appears to continue postflight; interestingly, higher preflight fitness levels and ongoing inflight exercise regimens may reduce this risk.[637] Furthermore, analyzing saliva and/or urine samples collected pre-, during and every other day postflight, Mehta *et al*. have shown that shedding patterns vary among the viruses:[633] EBV was shed by astronauts across all three phases, although at a significantly higher level during flight; VZV was shed during late flight and during early recovery (≤day five) only; CMV was shed during all three phases, with no significant difference among phases.[633] The persistence and differential patterns of postflight shedding have been confirmed by other groups, for EBV,[636, 638, 639] VZV[639, 646] and CMV[639] across all phases, including in the immediate and early recovery periods. However, there has been limited long-term postflight sampling that might provide better insight into the absolute temporal longevity of increased viral shedding; one group used recovery day 120 versus preflight values as a baseline in order to control for potential stress levels.[639] Significantly, and as described above (**see 6.2.1. *Immune-Associated Outcomes***), one interesting bed rest study used centrifugation to mimic reentry following 16 days of HDT bed rest. Stowe *et al*. observed, in samples taken at four hours after reambulation, significant increases in WBCs, PMNs, and urinary epinephrine, and a significant decrease in eosinophils compared to levels

immediately prior to centrifugation.[650] However, of the remaining scored bed rest publications, only three studies addressed time points within the first few days (2—6 days) following reambulation,[651, 674, 702] and all reported a return of assessed parameters to baseline levels.

As described previously (**see 6.1.3. *Animals in Space Category***), only one[684] of the scored rodent flight studies involved a long-duration flight (91 days), with the remainder being flown on missions in the range of eight to 35 days. The majority of scored studies performed a single sacrifice time point, taken at around 3—4 hours post-landing, *i.e*. within the first 24 hours of recovery, a lag time not dissimilar to the equivalent astronaut "immediate" sampling times. At the immune organ level, although we were unable to identify equivalent observations in astronauts, several studies reported decreased splenic and/or thymic mass at landing following spaceflights of 12 days,[681] 13 days,[680, 721] 30 days,[657] and 35 days.[722] Following 30-day flights, the thymus cell count has been shown to decline progressively through recovery day seven; the spleens also fail to return to baseline by recovery day seven, but without any further decrease from landing.[657] Unusually, following a 10-day flight, a single study reported an increase in rat body weight, but with no change in thymus mass.[658]

We were able to identify several rodent spaceflight studies that addressed immediate postflight WBC counts. Contrary to astronauts, who have appeared to consistently demonstrate an increased WBC count at landing, Pecaut *et al*.[679] and Lange *et al*.[723] saw no change in murine and rat WBCs, respectively, whereas Ichiki *et al*. reported decreased numbers of total leukocytes and absolute numbers of lymphocytes and monocytes in rats at landing, together with elevated neutrophils, despite seeing no changes other than neutrophilia inflight.[724] Pecaut *et al*. also have reported a slight (non-significant) decrease in murine WBCs relative to ground controls following a 12-day flight, as well as a significant decrease in peripheral monocytes.[679] There has been a focus on changes in splenocyte populations in many of the scored publications in this category, likely due their role in immune surveillance. Following a 10-day flight, Pecaut *et al*. saw a significant reduction in the percentages of total splenic T cells and CD4+ cells at landing, as well as a significant decrease in the CD11b+ neutrophil and macrophage population.[658] After a 13-day flight, Gridley *et al*. also saw a significantly lower percentage of T cells relative to ground controls, but a higher percentage of NK cells;[677] splenic lymphocyte, monocyte/macrophage and granulocyte counts were reduced.[680] Similarly, following both 13- and 14-day flights, significant decreases in CD4+ T cells have been observed.[725, 726]

Although we are unaware of direct testing of viral reactivation in spaceflight animals, as observed in astronauts, lymphocyte reactivity has appeared consistently altered in flown animals. In general, stimulated lymph node and/or splenic lymphocytes have demonstrated decreases in their interleukin (IL)-2,[677, 681, 683, 725-727] interferon (IFN)-γ,[681, 727] and IL-4 responses,[681] although without change in the tumor necrosis factor (TNF)-α response.[680, 681] However, in absolute terms, such responses have not been universal:[728] increased IFN-γ responses have been recorded;[677, 725] following 14-day flights, one study showed a significantly increased TNFα expression on landing, together with a significantly decreased IL-1 and TNFβ expression;[656] whereas, in an independent study, rat lymph node lymphocytes showed no change in their IL-2 response.[678] Furthermore, rat bone marrow and splenic NK cells have demonstrated decreased cytotoxic activity following a 14 day-flight, but only with respect to one of two target cell lines, suggesting a selective response.[729] Both bone marrow and splenic cells also displayed shifts in lymphocytic and myelogenous cell markers, although these shifts differed from those seen in matched HLU animals.[690] Interestingly, of the two scored studies that looked beyond the immediate post-landing effects, some of the assessed immune signals following a 30-day flight, *e.g*. splenic ph-RelA and thymic ph-IRF3, appeared to be altered only at a later recovery (day seven) time point,[657] whereas the other study demonstrated a return to baseline for all parameters by recovery day 14.[656]

Of the three scored ground-based studies that looked at post-suspension parameters, Paul *et al*. showed that, on reambulation day seven, although murine blood cell counts of WBCs, lymphocytes, monocytes, *etc*. were normalized to baseline, splenic leukocyte subpopulations continued to differentially express function-associated genes, suggesting persistently dysregulated immune and hematological systems.[666] Cao *et al*. saw a decrease in peripheral WBC and lymphocyte counts at the end of 28 days of murine HLU suspension, but again with restoration to control levels by recovery day seven.[649] However, more specifically with respect to the lymphocytes, they showed increases in CD4+ T cells, but decreases in B cells and NK cells; although the CD4+ T and B cell numbers recovered quickly (by day seven of reambulation), NK cells did not, and recovery for all types were linked to changes in bone marrow hematopoietic stem cells and their associated lineages.[649] In addition, Horie *et al*. reported that the observed reduction in thymic mass and cell count seen at the end of 14 days of HLU suspension persisted for at least three days following reambulation.[673]

6.2.4. *Immune-Associated Mechanisms*

For decades, authors have suggested that the observed changes in leukocyte numbers increase in parallel with the incidence of inflight stress, positing that stress, and not microgravity, is the major effector of these changes.[686, 687] The hypothesis of stress (versus microgravity) induction has been supported by a number of studies showing that astronaut monocyte[640] and neutrophil[641] responses are reduced not only post-, but preflight,[640] including their ability to phagocytose, elicit an oxidative burst and degranulate.[634] Furthermore, although monocyte numbers do not appear to change, neutrophil numbers have been seen to increase by ~85% at landing.[641] Interestingly, shuttle crewmembers have exhibited increased stress hormone levels and altered leukocyte subsets both prior to launch and at landing, with long-duration crewmembers exhibiting significantly greater spikes in both plasma and urinary cortisol at landing, supporting a role for mission duration on the magnitude of immune changes.[730]

As noted in the previous paragraph, a number of investigators, looking at cells isolated from various space (human and animal) models, have identified a compromised lymphocyte response to stimulating agents, as measured by decreases in IL-2 and/or IFN-γ production,[677, 708, 709, 720] supporting a decrease in T cell anergy and a blunting of the immune system.[651] In addition, an observed increase in salivary bacterial load likely reflects an overall weakening of the immune system since it has been shown to correlate with EBV reactivation.[630] However, again, the role of microgravity *per se* versus other stress factors in these observations is unclear. Certainly, stress-associated increases in glucocorticoids have been shown to reduce thymopoiesis, decreasing T cell production and offering an explanation for some of the lymph organ changes observed in HLU studies.[648] Importantly, and as noted earlier, systemic indicators of increased stress have been frequent observations across all human and animal immune-associated space models,[668, 669, 731, 732] but may be ascribed to various factors found within the space environment in addition to microgravity, including social[672, 733] and psychological stress,[734, 735] dehydration,[681, 731, 736] *etc*.

7. Overview, Discussion and Conclusions

As part of the development of this report, a literature search was performed focused on research studies addressing effects induced by exposure to microgravity. The principal goal was to compare the variously utilized *in vivo* microgravity models and identify those that may be most useful in risk assessment and/or countermeasure development. Searches were refined by critical endpoints seen not only as being among the chief potential risks to astronauts, but also considered to be greatest affected by microgravity: musculoskeletal; vascular (cardiovascular/cerebrovascular); CNS (physical, behavioral, ocular); and immune.

7.1. Overview of Microgravity Models: Specific Site/Endpoints

7.1.1. *Musculoskeletal Microgravity Models*

Overall, there is a commonality with respect to both bone and muscle loss as endpoints across all microgravity models (**Table 17**). Comparing specifically between the human space (astronaut) versus ground-based (bed rest) models, differences in bone endpoints appear to be a matter of degree, with the incidence rate and level of bone loss[141, 179, 737] and loss of urinary calcium[136, 138, 738] being lower in ground-based studies; muscle endpoints are generally similar across all models, with the exception of a proposed linear induction in muscle loss following HDT bed rest[291] versus non-linear seen in the other models. Interestingly, it appears that increased calcium excretion is a characteristic response seen in both human and animal microgravity studies, although its role in space-induced bone loss, other than as a secondary response to changes in the bone microenvironment, is unclear. Other factors in the bone-calcium homeostatic balance[739] also have been seen to be disrupted following bed rest, including decreased serum levels of parathyroid hormone[135, 740] and vitamin D,[141] although vitamin K appears to play a greater role as a countermeasure for mitigating bone turnover in space.[203] Of note, limb immobilization appears to generate greater loss of strength and volume in the specifically targeted muscles than does either bed rest or limb suspension.[264]

In general, it appears that the proposed mechanism of space-induced homeostatic disruption in the musculoskeletal microenvironment is recapitulated in human HDT bed rest, although definitive correlations are uncertain due to discrepancies in study lengths, inconsistently applied analytic techniques, and a lack of serial or comparative sampling conducted in either astronaut or HDT studies, preventing any “head-to-head” examination of the human models.

Indeed, of the scored astronaut studies, only two included a concurrent bed rest control group,[83, 84] and neither addressed specific bone or muscle changes. Importantly, the relatively limited observation that cortical bone loss might exceed trabecular bone loss during the first 60 days during human ground-based modeling[168] leads to questions regarding differential mechanism(s).

Differences in the relative magnitude and temporal induction of both bone and muscle effects in the space- versus ground-based human models highlight a lack of information on the interconnectivity between microgravity and other stress factors in the space environment, most notably radiation, a gap which may be critical given the known effect of radiation on bone[246, 741, 742] and muscle[743, 744] remodeling. Furthermore, the relative duration of ground-based unloading is an issue that requires significant additional interrogation in order to extrapolate findings across models, both intra- and inter-species. For example, although there is evidence that 28 days of unloading of skeletally mature rats induced equivalent changes in vBMD, bone mineral content and cortical area in the rat PTM[147, 745] to those seen in the human femoral head and proximal femur after 4—6 months of spaceflight,[189] it does not appear that the same level of directly confirmatory data are available for equivalency between human HDT bed rest and spaceflight. This highlights a potential incongruence when extrapolating data between "long-term" human models, *i.e.* between 6-month spaceflight and 60-day to 90-day bed rest.[76] The supposition of equivalence between these two periods appears to be based on an (unreferenced) observation of a lack of full trabecular BMD recovery at the hip following long-term bed rest[76] that also had been seen in a small astronaut study at 2—4.5 years following a six-month spaceflight.[77] It may indeed be true that, for this particular endpoint, the relative unloading duration in the two human models induces an equivalent effect, however, it is appears highly speculative that 60—90 days of long-term bed rest can act an analog for six months of spaceflight across all musculoskeletal changes given the differential changes seen in terms of incidence, severity and time line, and even more speculative when applied to other systems. Nonetheless, it is important to acknowledge that, as a result of the mechanistic findings seen across the human studies, both space- and ground-based, exercise[85, 128, 195, 200] and nutrition[84, 195] regimens have been introduced into space programs, and bone formation-promoting pharmaceuticals have been assessed.[85, 200, 746] These countermeasures have met with an appreciable level of success with respect to astronaut musculoskeletal outcomes,[85, 747] although the increased risk of fracture remains.[178]

Table 17. Overview of musculoskeletal microgravity model characteristics.

Model	Bone loss	Induction	Recovery	Mechanisms		
Astronaut	Trabecular loss > cortical loss in weight-bearing bones	~0.8—3%/month Rapid to day 20; plateau	Density/strength >1-year postflight	↑↑ loss of urinary Ca	↑ osteoclasts/bone resorption markers ↑ early to ~100%; ↓ osteoblasts/bone formation ↑ after 1 month to ~7% per month	Disrupted bone homeostasis
Human – ground-based	**Possible cortical loss > trabecular loss in weight-bearing bones over first 60 days**	**0.3—1% loss/month** Rapid over 1—2 weeks; plateau	Bone recovery can take up to 5-6x longer than unloading period	↑ loss of urinary Ca	↑ osteoclasts/bone resorption ↑ early to **~50-75%;** ↓ osteoblasts/bone formation markers **no change**	Disrupted bone homeostasis
Animal – space	Trabecular loss > cortical loss in lower/hind limbs	unknown	**Relatively rapid recovery over 30 days postflight**	unknown	**Rats: ↑ osteoclasts/bone resorption only** Mice: ↑ osteoclasts/bone resorption; ↓ osteoblasts/bone formation	R**ats: Disrupted growth** Mice: Disrupted bone homeostasis
Animal – ground-based	Trabecular loss > cortical loss in lower/hind limbs	Rapid loss over 1—2 weeks	**Density/thickness up to 3x longer than unloading period**	↑ loss of **fecal Ca**	↑ osteoclasts/bone resorption; ↓ osteoblasts/bone formation	**Rats: Disrupted growth** Mice: Disrupted bone homeostasis
	Muscle loss	**Induction**	**Recovery**	**Mechanisms**		
Astronaut	↓ postural muscle volume ↓ strength vs. muscle area/volume	Rapid (≤1 week) loss of volume/ strength; non-linear induction; mission-length dependent	Rapid early recovery, but persistent damage; recovery mission-length-dependent	Atrophy: ↓ skeletal muscle protein synthesis; ↑ skeletal muscle protein degradation possible		
Human – ground-based	↓ postural muscle volume ↓ strength vs. muscle area/volume **↑ deposition muscle adipose tissue**	Rapid (1—2 week) loss of volume/ strength; **possible linear induction**	Rapid early recovery; **potential overshoot (hypertrophy)**	Atrophy: ↓ skeletal muscle protein synthesis; ↑ skeletal muscle protein degradation possible		
Animal – space	↓ postural muscle volume	Rapid (≤1 week) loss of volume/ strength; non-linear induction	?	Upregulation of negative regulators of skeletal muscle homeostasis, *e.g.* myostatin		

Animal – ground-based	↓ postural muscle volume	Rapid (≤1 week) loss of volume/ strength; non-linear reduction	Rapid and complete restoration	Atrophy: ↓ skeletal muscle protein synthesis; upregulation of negative regulators of skeletal muscle homeostasis, *e.g*. myostatin

bold font indicates differing response to space/human equivalent

Comparing the rodent space- versus ground-based models, microgravity musculoskeletal characteristics appear similar with the exception of a potential species difference with respect to growth failure (rats) versus homeostasis dysregulation (mice).[227] However, Vico *et al*. performed a "head-to-head" 7-day spaceflight versus HLU study in rats, and the differential findings between the two groups led the authors to conclude that mechanisms of bone loss in space likely differ from those of ground-based studies.[748] Furthermore, as seen in the human studies, definitive correlations between the mechanisms of bone loss experienced in space- versus ground-based rodent studies are uncertain due to discrepancies in study lengths, inconsistently timed endpoints and analytic techniques, and a lack of serial or comparative sampling. Nonetheless, as described in a recent review, the HLU model has allowed for significant insight into specific aspects of the bone microenvironment, in particular the roles played by osteoblasts, osteoclasts and osteocytes in disuse-associated bone loss.[227]

7.1.2. *Vascular Microgravity Models*

Vascular changes, in the form of peripheral vessel, cerebrovascular and cardiac remodeling, have been frequent indicators of adaptation to reduced gravitational conditions, seen in both space and ground-based models.[332, 379, 468] Fortunately, other than aerobic capacity and the postflight effects of orthostatic intolerance, the likelihood of occurrence and operational impact of such risk factors appears to be relatively low.[342, 414] Nonetheless, the multiplicity of physiological changes and the potential for more systemic impact requires the development of appropriate tools and countermeasures, and the utilization of appropriate models would abrogate the limited data from astronaut studies. However, as is evident from **Table 18**, there is significant heterogeneity in the absolute changes seen in vascular parameters across all four models, notably between the human space- and ground-based models. The reduced myocardial workload experienced under both conditions appears to result in cardiac remodeling, although it is not consistently clear that equivalent functional changes result.[421] For example, in a loosely-matched 6-week HDT bed rest study versus a 10-day spaceflight, trends towards reduced LV mass were seen in both cohorts,[379] whereas in a slightly higher powered comparison between ~160 days spaceflight versus 70 days of HDT bed rest, astronauts demonstrated lower pre- versus postflight increases in plasma volume and heart rate versus bed rest subjects, as well as a postflight trend towards an increase in mean

Table 18. Overview of vascular microgravity model characteristics.

Endpoint	**Astronaut**	**Human ground****	**Animal – space**	**Animal - ground**
Fluid redistribution (lower to upper limbs)	Y	Y	Y	Y
Plasma volume	↓	↓	↓	? / no change
Cardiac output	↑	↑/↓	?	?
Heart rate	↓	↑/↓	↑	**↑ rats;** ↓ mice
Stroke volume	↑	↑/↓	?	?
Left ventricle mass	↓	↓/no change	?	↓
Blood pressure	↓	↑/↓	no change	**↑ rats;** no change mice
Mean arterial / central venous pressure	↓	↓	?	?
Vascular resistance	↓	↑	↓	↓
Cerebrovascular flow	↑	↑/↓/no change	↑	↑
Intracranial fluid shift/ change in brain volume	Y	Y	?	?

bold font indicates differing response to space/human equivalent
Y (yes) present
? insufficient data
** Faster onset and higher response seen in majority of parameters following dry immersion versus HDT bed rest
.

arterial pressure versus a decrease following bed rest.[256] This again highlights the incongruence discussed in **section 7.1.1.** regarding the value of ascribing equivalence between "long-term" bed rest lasting 60—90 days versus spaceflights of 4—6 months, especially if using such data for risk estimation.

Differences in even basic vascular parameters may be the result of the exquisite sensitivity of the microcirculation to subtle changes in gravity;[749] for example, in ground-based studies, gravity continues to contribute to intra-thoracic pressure not experienced in space,[422] and there are differential levels of muscle sympathetic nerve activity between the two conditions, affecting vascular resistance and dilatation.[19] Furthermore, there appear to be rapid fluid shifts from the vascular to interstitial spaces during weightlessness, notably into the thoracic cage and muscles,[750] which are not recapitulated at ground level. Nonetheless, although levels of effect and the temporal sequence of events during bed rest do not necessarily coincide with those seen in astronauts, such differences may be wholly or partly dependent on the time of observation during the period of weightlessness and/or immobilization,[412] since a paucity of overlapping time points between the models, both human and animal, and the lack of direct model controls limits direct comparisons in all cases.

7.1.3. *CNS Microgravity Models*

Comparison of the various microgravity CNS models is hampered by limited systematic analyses and a lack of inter-model studies. Indeed, we identified only a single publication that directly compared human spaceflight to bed rest subjects,[751] although the periods of unloading differed significantly between the cohorts (70 days HDT bed rest versus 13 or 160 days spaceflight) and compared only pre- versus post-analysis of balance control. Overall, structurally, it appears that many of the CNS physical changes described during and following spaceflight have not been reported in either human ground-based or animal studies (**see Table 19**). Interestingly, some of the putative effects ascribed to astronauts are inferred from symptomology rather than direct measurements, *e.g*. proposed microgravity-induced increases in ICP due to overlapping symptoms in SMS versus intracranial hypertension.[582, 620, 752] Nonetheless, whether discrepancies are due to the paucity of sufficient comparative astronaut data, incomplete removal of gravitational effects or the failure to include other spaceflight stressors (*e.g*. space radiation, sleep disturbance, *etc*.) in the ground-based studies is unclear. Thus, it is important to emphasize the care that is needed when

attributing CNS-related impairments to microgravity alone given the known impact of competing stressors found in the space environment, such as sleep fragmentation,[753, 754] circadian rhythm disruption,[755, 756] *etc*.[605, 757, 758]

Although ground-based human modeling might have allowed for a better understanding of the temporal progression and underlying mechanisms of space-induced CNS effects, we are aware of few systematic, sequential analyses that would enable determination of initiation, flexion points, interim changes, *etc*. Indeed, drawing conclusions from bed rest studies with respect to the time line of CNS events is fraught with uncertainties since the timing of assessments appears random and non-sequential, with many of the scored studies including a countermeasure or additional stressor (CO_2) arm, and none including ambulatory controls, so that all comparative changes were determined against a pre-unloading baseline. Significantly, the timing of analyses in these studies may be critical; for example, when determining both the temporal progression of IOP and ICP under microgravity conditions and their relationship to consequent visual disturbances,[591] the rapid rise in IOP appears relatively transient, normalizing within the first few days of flight and/or bed rest,[591, 759] so that any delay in sampling points may miss relevant changes. Furthermore, since the majority of astronaut/cosmonaut assessments are single point analyses, conducted postflight at varying time intervals after reentry, it is difficult to distinguish recovery kinetics from inflight effects. Additionally, results within the majority of studies are pooled despite a significant time spread among sample acquisition points; for example, Roberts *et al*. grouped the observed MRI changes from a cohort of astronauts sampled over a postflight period of 1—20 days.[433] Similarly, even in those human ground-based studies where multiple assessment points were performed following bed rest,[517-519] data tended to be merged into a "post" group, with little discussion regarding temporal kinetics.

Drawing comparisons between space-flown versus ground-based rodent CNS models is equally, if not more, tenuous. Again, we only identified a single study that compared the two model paradigms;[628] a comparison was performed at the data level alone, *i.e*. conditions were not matched, and looked at nonrandom gene expression post-unloading, with the choice of genes dependent on previous postflight analyses. Systematic analyses of structural, neurovestibular/ neuroocular, or sensorimotor/cognitive changes have not been performed between spaceflight versus ground-based models; nonetheless, there appears to be only limited correlation between the outcomes seen in the corresponding human versus rodent models (**see Table 19**).

Table 19. Overview of CNS microgravity model characteristics.

Endpoint	Astronaut	Human ground**	Animal – space	Animal - ground
Postflight:				
ventricular brain volume	↑	↑ possible	No change	↑ possible
white matter volume	↑			
gray matter volume	↓			
CSF volume	↑			
SMS	Y	Y (no GI)		
IOP	↑ inflight	↑	↓ postflight	
ICP		↑		
SANS:				
Optic disk edema	Y	Y possible		
Choroid folds	Y	Y		
Retinal thickness	Y	Y		
Visual acuity	Y			
Hyperopic shifts	Y			
Globe flattening	Y			
Sensorimotor adaptation	Balance and gait disturbance	Balance disturbance	Gait disturbance	
Cognition	Gradual ↓ reaction time			
	↓ accuracy	**↑ accuracy**	↓ learning	↓ learning
	↓ attention		↓ memory	↓ memory

bold font indicates differing response to space/human equivalent
Y (yes) present

7.1.4. *Immune Microgravity Models*

Overall, it appears that changes seen in immune cell populations during short-term flights persist; the long-term effects seen in CD4+, CD8+ and NK cells likely contribute to astronauts' inability to suppress the observed virus reactivation, increasing in frequency and amplitude with mission length.[697] Indeed, 53% of shuttle and 61% of ISS astronauts have been shown to shed one or more herpes viruses in either their urine (CMV) or saliva (EBV, VZV, HSV-1).[697] However, these findings contrast with those seen in human ground-based studies (**see Table 20**), many of which have failed to demonstrate any significant changes in immune function in terms of changes in CD8+ subsets, latent viral reactivation, *etc*.[705, 707] Furthermore, despite evidence of significant and rapid temporally differential findings, the data in many of the scored immune microgravity studies from astronauts from both short- and long-duration missions have been pooled. For example, Crucian *et al*. looked at 27 astronauts following a range of short-duration flights (10—18 days).[720] Such pooling, although potentially allowing for better statistical analysis, may well obscure critical

changes in an immune system that has evolved to respond both acutely and chronically to stress.[760, 761]

Table 20. Overview of immune microgravity model characteristics.

Endpoint	Astronaut	Human ground**	Animal – space	Animal - ground
Differential T cell subset alterations	Y pre, in- and postflight	Y ↑ modest		Y early mid-HLU
WBC numbers	↑ postflight		**No change**	↑ early mid-HLU
PMN numbers	↑ postflight	↑ post-HDT	↑ postflight	↑ post-HLU
Granulocyte numbers	↑ postflight		**↓ postflight**	↑ post-HLU (NS)
NK cells	↑ late inflight/no change		↑ postflight	
B cells	No change		No change	**↓ splenic B cells**
Splenic/thymic mass			↓	↓
T cell function	↓ postflight		↓ postflight	↓ post-HLU
NK cell function	↓ postflight		↓ postflight	
Viral shedding	↑ pre, in- and postflight	**No change**		
Stress hormones	↑ pre, inflight			↑ mid-HLU

bold font indicates differing response to space/human equivalent
Y (yes) present

Interestingly, overall, a relatively high level of congruence was observed between the human and rodent models described in this report (**see Table 20**). For example, similar to astronaut findings, splenic and liver tissues from mice sacrificed at day 21—22 inflight indicated no effect on B cell repertoire compared to matched ground controls.[655] Similarly equivalent to the human immobilization model, a number of HLU studies have failed to demonstrate any appreciable effect on the immune system other than those that may be attributable to the stress of the apparatus.[762, 763] Indeed, stress-induced loss of body weight, reduced food and water consumption and increased catecholamine levels have been consistently observed in HLU models.[668, 764] Furthermore, Tahimic *et al*. showed a significant reduction in CD4+ T cells following HLU in singly, but not group, housed animals, although HLU appeared to increase plasma corticosterone levels in both isolated and socially housed groups.[672] These findings again emphasize the need to isolate potential stressors in observed effects. A pivotal study, conducted by Pecaut *et al*., compared space-flown rats to HLU, and also included a group (L+HLU+L) that underwent centrifugation prior to and post-suspension as an analog to launch and reentry, all with a delayed 3-hour sacrifice time point.[658] Tellingly, although the centrifugation group was the closest to recapitulating space-

induced immune changes versus either the HLU alone or vivarium cohorts, all of the ground-based models failed to induce equivalent immunological changes.[658]

A number of the scored publications were identified as performing comparative model studies. For example, one study compared data samples (blood) from astronauts and rodents (HLU);[649] however, the limited overlap in experimental conditions and differential endpoints prevented concrete conclusions being drawn. Nonetheless, a small number of the scored spaceflight rodent studies included a matched ground-based HLU cohort, providing an opportunity to directly compare the space- versus ground-based animal models across equivalent endpoints (**see Table 21**). As should be apparent, the findings from the HLU models differed significantly from those reported for the flown cohorts for almost every value described.

Table 21. Direct comparisons between rodent spaceflight and ground-based immune microgravity models.

Endpoint	Space	Ground (SYNCH) synchronous	HLU	Vivarium (VIV)
Spleen mass[680]	**↓ postflight † vs. HLU**	–	Normalized to VIV	Baseline
Thymus mass[680]	**↓ postflight ** vs. HLU**	–	Normalized to VIV	Baseline
Liver mass[680]	**↓ postflight † vs. HLU**	–	Normalized to VIV	Baseline
Body mass[658]	no change vs. VIV	–		Baseline
WBC cell count[680]	**↓ postflight * vs. HLU**	–	Normalized to VIV	Baseline
Lymphocyte cell count[680]	**↓ postflight *** vs. HLU**	–	Normalized to VIV	Baseline
T cells (TCR+)[658]	**↓ postflight † vs. VIV**		No change vs. VIV	Baseline
T cells (CD4+)[658]	**↓ postflight ** vs. VIV**		No change vs. VIV	Baseline
Monocyte/macrophage cell counts[680]	**↓ postflight ** vs. HLU**	–	Normalized to VIV	Baseline
CD11b+ macrophage[658]	**↓ postflight * vs. VIV**		No change vs. VIV	Baseline
Granulocyte cell counts[680]	No change vs. HLU	–	Normalized to VIV	Baseline
LPS-induced cytokines:				
TNFα[680]	**↓ postflight *** vs. HLU**	–	Normalized to VIV	Baseline
IL-10[680]	**↑ postflight ** vs. HLU**	–	Normalized to VIV	Baseline
IL-6[680]	**↑ postflight * vs. HLU**	–	Normalized to VIV	Baseline
Lymph node lymphocyte IL-2 response[678]	**15-fold ↑**	15-fold ↑	**21-fold ↑**	10-fold ↑
Splenic NK cell activity (YAC-1 target cells)[729]	**↓ postflight ** vs. HLU**	No change vs. VIV/ HLU	**No change vs. VIV/ SYNCH**	Baseline
Splenic NK cell activity (K-562 target cells)[729]	**↓ postflight ** vs. HLU**	No change vs. VIV	**↑ ** vs. VIV/ SYNCH**	Baseline
Bone marrow cell response to GM-CSF (colony #)[690]	**1.3 ± 0.4 vs. VIV**	7.4 ± 1 vs. VIV	**12.4 ± 4 vs. VIV**	Baseline

bold font indicates differing responses between space and ground-based equivalents
Y (yes) present

† $P > 0.01$ (trend)
* $P > 0.05$ (significant)
** $P > 0.001$
*** $P > 0.005$

7.2. Discussion of Specific Microgravity Models

7.2.1. *Astronaut Microgravity Models*

Within the space physiology field, direct examination of the changes seen in the astronaut cohort might reasonably be considered the gold standard for analysis; however, it is important to appreciate the "cons" of using this approach:[765]

- The astronaut cohort as a whole is relatively small (only ~600 people have reached space to date),[766] and the number of astronauts available for sampling per mission is naturally smaller, making any resultant data set difficult to interpret. Although pooling the data from multiple missions has the potential to provide greater statistical power through interrogation of specific endpoints within a large cohort, especially since this allows for the inclusion of a matched control group (*i.e.*, astronauts that have not flown), this approach has been employed in very few studies.[342, 535]
- The variables in each potential astronaut dataset are considerable, including: age range (30—57 yrs; median 44 yrs);[767] gender mix (only ~11% female);[768] length of mission (ranging from days to months). Psychological, psychosocial, and habitat stressors, as well as other human factors, differentially affect astronauts with respect to their health and productivity, dependent on the mission and crew makeup, *etc*.[449] Furthermore, ethical concerns require that proven countermeasures cannot be withheld, limiting the ability of researchers to generate appropriate control populations.[239]

With specific respect to determining the effects from microgravity, many of the symptoms observed in returning astronauts are considered highly correlative to those seen following long-term bed-rest.[24, 76, 179, 769, 770] As a result, many early researchers assumed that the main cause of the observed adaptive changes were due to disuse and/or a physiological response to the shift in gravitational force on the human body.[16] However, as noted in the **Introduction**, astronauts are exposed to an environment that includes a wide range of significant physiological stressors in addition to microgravity,[182] some of which may, individually or in combination with microgravity, generate systemic changes.[9, 771, 772] Stressors include, but are not limited to, space radiation,[771, 773] a suboptimal diet resulting in an altered gut biome,[774] limited exercise,[775] social isolation,[767, 776, 777] and others.[10, 778] As a result, since an overwhelming majority of studies performed on the astronaut cohort have made use of internal controls only (*i.e.*, comparing pre- versus postflight samples), such studies offer little means of differentiating effects due to microgravity alone, as

distinct from the overall response to the complex space environment. Unfortunately, as described in the 2017 NASA Evidence Report HRP-47072,[239] following the fatal fire on Apollo 1, plans to perform inflight measurements of critical parameters were changed, such that the majority of astronaut studies performed subsequently have, indeed, only included pre- and postflight samples. It is further likely that the use of immediate postflight sampling to assess end-of-flight changes may have skewed results dependent on the endpoint being measured. For example, although bone loss *per se* is unlikely to undergo a speedy normalization following a return to gravity, endpoints such as changes in the immune system are inherently adapted to a rapid response, so that use of markers or other values that have been affected by the stress of reentry or are susceptible to rapid readaptation may have introduced significant errors in risk estimation.

7.2.2. *Human Ground-Based Microgravity Models*

Because of the putative overlap among many of the respective outcomes seen post-spaceflight with those seen after bed rest, and to a lesser extent dry immersion, these specific models have become the most widely used analogs of microgravity, especially with respect to addressing musculoskeletal and cardiovascular changes.[24] Indeed, although many of the musculoskeletal effects seen during and following bed rest are less intense than those identified following spaceflight, the mechanisms underlying the changes have been presumed to be similar.[157] Nonetheless, it is apparent from this report that discrepancies remain between effects seen in space versus those seen in ground-based models, partially, although not wholly, due to differential patterns of fluid distribution.[779] For example, there appear to be rapid fluid shifts from vascular to interstitial spaces during weightlessness, notably into the thoracic cage and muscles,[750] which are not recapitulated at ground level. Furthermore, during bed rest, gravity continues to contribute to intra-thoracic pressure, not experienced in space,[422] and there are differential levels of muscle sympathetic nerve activity between the two conditions, affecting vascular resistance and dilatation.[19]

In order to determine effects attributable to microgravity alone, the conditions described in the **Introduction** and **7.2.1.** should to be more adequately recapitulated:

- Physical: A common disadvantage across ground-based human (and animal) models of microgravity is researchers' inability to simulate the complete range of physical factors found in the space environment, *e.g*. low dose/low dose rate space radiation, high CO_2

concentrations, altered day and night cycles, *etc*. Attempts have been made to compensate for this deficiency, with a number of the scored studies published since 2017 describing experimental conditions including altered CO_2 levels.[353-356, 396, 517-522, 545] In addition, in order to address this issue further, NASA and the European Space Agency have developed the :envihab facility, located in the German Aerospace Centre in Cologne, Germany,[780] where environmental factors such as ambient light, temperature, humidity, and O_2 and CO_2 levels can be controlled and a short-arm centrifuge is available.[781]

Many basic astronaut physical characteristics also have not been well addressed in the ground-based human studies, such as age, sex and physical fitness. For example, the majority of the participants in human bed rest studies are younger than the astronaut cohort (average age of ~33 years in immobilization studies vs. ~43 years in the astronaut studies). Although females make up a smaller percentage of the astronaut cohort compared to males, they similarly make up a smaller percentage of ground-based studies (~18% of subjects in our surveyed database). This smaller percentage is despite the multiple indications of potential gender-specific responses in areas of interest that should be appropriate for investigation in ground-based studies.[422, 782, 783] Finally, astronauts undergo vigorous physical training and/or assessment, whereas human bed rest study participants are rarely matched at this level, likely leading to uncertainties, particularly with respect to variations in observed musculoskeletal and cardiovascular outcomes.

- Dietary: Early observations of increased calcium excretion in astronauts and its correlation with a high salt intake have indicated that diet may be a significant issue in space aeronautics.[784] Not surprisingly, therefore, the majority of human ground-based studies have been conducted with a controlled dietary intake, although many such controls have been used as a means of maintaining essential physiologic elements, body weight, *etc*.[769] It may be worth noting that some of the dietary supplements assessed as potential countermeasures in bed rest studies have proved less than optimal,[95, 317] emphasizing the need for better models for countermeasure assessments prior to deployment in space.
- Psychosocial: The psychosocial effects of isolation and confinement on astronauts is a spaceflight factor of interest.[776] Although we are aware of few direct observations on the effects of social stress on bed rest subjects,[785] its role as a factor in the development of other systemic outcomes does not appear to have been pursued in depth.

7.2.3. *Animals in Space Microgravity Models*

When considering the relevance of animal models to the human condition, it is important to note that participants in studies in space are exposed to the majority of the same stressors experienced by astronauts:

- Physical: Hypergravity (during launch and re-entry), microgravity, space radiation and altered CO_2 levels;
- Dietary: Some of the same constraints on astronaut diets also apply to rodents, including limited crumbling, adequate shelf stability, and compatibility with on-board delivery systems.[786] Significantly, environmental stressors experienced during flight are known to affect food bioavailability,[787] so that the composition of rodent spaceflight diets has undergone several iterations, and their current and future development is a relatively high NASA priority.[786]
- Psychosocial: Astronauts are believed to be affected by their isolation and confinement.[776] Mice are highly social animals with a strong hierarchical structure and separation can induce depression, particularly in females.[788] The MDS separates the animals, as described above, whereas the RRHS involves group housing; of note, there is a differential in numbers between the RRHS transporter and the on-board habitat necessitating a step-down from groups of ten to five, although this process does not appear to initiate excessive aggression in either males or females.[789] Although far from an optimal analog of human psychosocial stress,[790] nonetheless only those animal space studies which explicitly describe individual housing were scored as potentially recapitulating the psychosocial aspects of the astronaut space environment.
- Experimental: Adequate and appropriate controls: One important experimental consideration has been that, whenever possible, animal space studies have included both vivarium and ground-based control groups, the latter housed in identical modules as those used in space.[791] These ground modules are placed in an environmental chamber that mimics some of the conditions experienced during spaceflight (temperature, humidity, O_2 and CO_2 partial pressure, etc.), thereby controlling for stress induced by housing in the module itself.

Although the above may be considered positive factors of simulation, there are several elements imposed on the experimental design in animal space research which detract from the quality of the data and their utilization in risk estimation:

- Appropriate sampling/time points: One important feature of animal space (and ground-based) studies is to distinguish between inflight versus postflight sampling since, like humans, animals can undergo rapid readaptation upon reentry, and multiple hours frequently elapse while animals are being handled postflight.[29]
- New animal housing and payload systems have been built on ISS enabling the ability for on-board manipulations, such as dissection and tissue fixation. However, due to heightened mission complexity, few animal space studies have involved inflight sample collection, despite the potential for additional insight to inflight adaptations that cannot be deciphered from pre- and postflight sampling alone.
- Sample size: Overall, it is apparent that only a limited number of animals can be housed in space at any one time,[29] resulting in small group sizes. This issue is frequently exacerbated by two-armed experiments investigating countermeasures, such as specific diets, drugs, exercise, *etc*., further diminishing group sizes.

7.2.4. *Animal Ground-Based Microgravity Models*

One advantage to the use of ground-based animal versus human models is the ability of a researcher to impose experimental conditions that would be considered unethical in a comparable study in healthy humans.[792] However, recapitulation of the conditions described in **7.2.1.** as components of ground-based experimental design has been variable:

- Physical: Astronauts outside of LEO are exposed to two cosmic sources of ionizing radiation: energetic protons associated with SPEs and background GCR.[793] Although recent technical developments at Brookhaven National Laboratory have opened up the possibility of exposing rodents to a relatively realistic simulation of GCR,[563, 564] long-term logistical difficulties of creating such a simulation have led to the majority of combined-stressor space studies being performed using HLU with either low linear energy transfer gamma/X-rays,[173, 794] protons,[152, 562, 665] or single heavy ion[149, 152] exposures, the latter two being relevant to SPEs and GCR, respectively. The potential risks from space radiation alone[773, 795-797] or in combination with microgravity[46, 331] have been reviewed by many, and

so will not be addressed further in this report, but should be considered as a factor in ground-based space paradigms. Other physical environmental factors that may affect astronauts, such as increased CO_2, sleep deprivation, altered light-dark cycles, and steady-state noise, have rarely been included in ground-based simulated microgravity studies. One group described the use of a simulated long-duration spaceflight environment that included HLU, isolation rearing, steady-state noise, and altered light-dark cycle;[614] however, the group focused their analysis on cognitive changes alone, so it is unclear how well this model mimicked the astronaut environment across the broader spectrum of outcomes. The failure of rodent models to recapitulate fluid shifts seen in humans may be critical.

- Dietary: Diet composition has rarely been considered in the experimental design characteristics of ground-based research other than when being incorporated into a counter-measure study. However, one early study did consider the effect of a "space diet" on HLU animals with respect to musculoskeletal changes, and determined that there was little to no impact.[798] Whether this is true across other areas of interest is unknown.
- Psychosocial: As discussed in **7.2.2.** and **7.2.3**., the psychosocial effect of isolation and confinement on astronauts is a spaceflight factor of interest.[776] In the majority of earlier ground-based studies, the mechanics and jig constraints of both HLU and PWB meant that the subject rodents were housed individually, thereby potentially simulating the social stress experienced by astronauts. However, more recently, some groups have introduced paired housing into their HLU model;[58, 231, 528] of note, and as described in **1.1.3.** ***Animal studies in space***, AEM rodent residences in space involve group housing. In order to determine the impact of isolation on the outcomes associated with simulated microgravity, Tahimic *et al*.[672] performed a side-by-side, systematic assessment of unloaded (HLU) versus normally loaded animals housed either individually or in pairs. Importantly, they found that the musculoskeletal effects induced by HLU were unaffected by social isolation, although there were differential immune and adrenal responses, possibly associated with hippocampal changes.[532]

7.3. Final Conclusions/Thoughts

Why are models of biological conditions developed? In general, beyond philosophical curiosity, their function is to understand the etiology of a disease or adverse effect, usually with the intent to prevent its occurrence and/or develop treatment. But with specific reference to space, an additional use for biological models is to provide both information and data that can be used in the development of models for risk estimation. In order to accomplish any of these goals successfully, the biological models must not only display the same symptomology as the target disease or condition, but develop such through comparable biological mechanisms. Although variability in time lines is possible (and indeed likely) between models of different species due to temporal variations that occur dependent on body size, metabolic rate, *etc*., nonetheless, the basic underlying mechanisms must be equivalent. Therefore, a fundamental necessity when researching the effects of microgravity on the astronaut body is a clear understanding of the etiology, mechanism and progression of each endpoint of interest. Indeed, the relevance of an animal model response to human health is best determined only if the molecular pathogenesis is well understood in the human.[799] Therefore, the granular nature of animal model research requires that the cellular/molecular/signaling changes observed must be directly linkable to relevant human physiological deficits.

However, as outlined in **7.2.1**, the available inflight astronaut data can be described as heterogeneous at best, with inadequate and varying sampling times, derived from small and mixed populations in terms of even the most basic parameters, such as age, gender ratios, flight experience, providing little to no ability to determine effects from a single stressor, *i.e*. microgravity. Furthermore, a significant gap, evident throughout this report, is a fundamental lack of postflight recovery data across all endpoints of interest, especially with respect to long-term effects, thereby placing severe limitations on the ability of risk modelers to correlate pre-, in- and postflight biomarkers and/or risk factors with chronic outcomes. There is, therefore, a critical need to generate and maintain a bank of systematically acquired, multi-media samples, expanding on the work of the NASA GeneLab (see https://genelab.nasa.gov); importantly, samples should be acquired not only from astronauts, but also from appropriate and matched control cohorts. In addition, rationally-designed ground-based studies are needed in order to perform the necessary cross-validation among models across equivalent time lines, using the same technologies, and across multiple institutions.[800] Only through broad investigator access and interrogation, including

but not limited to multi-omics platforms, can statistically sound databases of information be developed.

Although this report essentially indicates that no single model completely recapitulates the array of in- and postflight conditions exhibited by astronauts, there appears to be mechanistic overlap among some models with respect to specific and various elements of each endpoint. Therefore, a clear understanding of the pros, cons and limitations of each model, achieved through the cross-validation studies suggested in the previous paragraph, would enable justifiable, albeit relatively tightly focused, experiments to be designed. Indeed, as noted in a recent review,[629] terrestrial analogs may be used to mimic specific stressors associated with spaceflight, although significant care must be taken if the goal is to isolate effects from a single stressor, *e.g*. microgravity; given the complexity of the space environment and the putative contribution to each endpoint from an array of stressors, this will be a formidable task.

However, from a personal perspective, it may be worth considering whether there is a need to identify effects from a single stressor for all endpoints. Indeed, a cursory scan of the tables in each section in this report suggests that, with few exceptions, non-astronaut studies that included one or more stressors in addition to simulated microgravity appear to result in an exacerbated response. Although we are unaware of any claims of synergism (or even additivity), it seems unlikely that the major space environmental stressors induce each endpoint of interest through the same mechanism(s), *e.g*. muscle loss induced by both microgravity and GCR occurring as a result of fluid redistribution. Therefore, it is likely that most, if not all, space-induced outcomes are a response to a heterogeneous combination of injuries. Furthermore, a relatively consistent observation from space-related physiologic studies is a homeostatic disruption of a plethora of systemic and/or cellular microenvironments (*e.g*. systemic immune status,[428, 642, 801, 802] microbiome,[428, 803, 804] vascular autoregulation,[805] bone[806] and bone marrow,[807] versus cellular metabolism,[428] mitochondrial homeostasis,[808] and calcium levels,[809] *etc*.). This observation raises the possibility that the development of countermeasures may be more efficiently accomplished if investigators target their research towards reestablishing homeostasis rather than focusing on outcomes from individual single stressors, a goal that may be more readily and efficiently achieved using the available models.

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